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CONTENTS

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AN INVESTIGATION OF THE ETIOLOGY OF MUMPS*

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PLATES 1 TO 3

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The earlier investigations concerning the etiology of mumps dealt with the cultivation of microorganisms by ordinary bacteriological methods. Cocci were isolated and cultivated from the aspirated fluid of the swollen glands, from the blood, and from the saliva; but attempts to reproduce the disease in experimental animals by inoculation of these bacteria into the parotid glands always met with negative results.

Granata in 1908 was the first to report attempts to reproduce the disease in animals by introducing into the glands material from the patient (1). He was able thus to produce in rabbits a rise in temperature of 3 days duration by injecting intravenously a bacterially sterile filtrate of saliva of patients suffering from mumps. He was also able to produce swelling of the parotid glands of rabbits by the direct inoculation of the gland with the filtrate. Granata therefore suggested that the etiological agent of epidemic parotitis is a filterable virus.

Nicolle and Conseil in 1913 produced swelling of the parotid in one of three monkeys by injecting directly into the parenchyma of the gland material aspirated from the parotids of children suffering with the malady. The other monkeys developed only a fever of 4 to 7 days duration and a mononuclear leucocytosis after an incubation period of 16 to 30 days (2).

Gordon in 1914 used a bacteria-free filtrate of saliva from patients with mumps for intracerebral inoculation of ten monkeys, five *Macacus rhesus* and five *cynomolgus*. Four of these animals, three of which were *Macacus cynomolgus*, died after having developed meningeal symptoms on the 4th day. They were found to have had a sterile lymphocytic meningitis, degenerative changes in the cortical neurones and anterior horn cells. Unsuccessful attempts were made to transfer the infection from one monkey to another by the use of filtrates of emulsions of brain and spinal cord. One monkey, inoculated intraperitoneally and intravenously, became ill after an incubation period of 7 days and showed swelling of the parotid glands (3).

Wollstein in 1916, using cats as the experimental animal, having observed that they gave more promising results than rabbits and monkeys, inoculated the parotids and testicles with filtered saliva which induced, after an incubation period of 5 to 8 days, a parotitis characterized by congestion, interstitial edema, mononuclear interstitial infiltration, and an orchitis characterized by degeneration of the epithelium with interference with spermatogenesis ("spermatorrhexis"), and an inconstant swelling and multiplication of the interstitial cells with cellular invasion between the tubules. She used a bacterially sterile filtrate of saliva of patients ill not longer than 3 days with the disease. By incubating, at 37°C. for 2 hours, a mixture of an emulsion of a gland removed at the height of infection, with immune serum obtained from a cat which had recovered from the infection, she was able to decrease the reaction of the cat to the inoculation. The virus increased in virulence through the fourth generation but rapidly decreased after the sixth transfer; and in only one instance were the effects observed at the eighth transfer. The virus remained virulent after 4 months of storage in 50 per cent glycerine (4). In 1921 Wollstein reported the production of an aseptic meningitis in cats by the intracerebral inoculation of bacterially sterile filtrate of saliva from patients ill with epidemic parotitis (5).

Kermorgant in 1925 reported that he had been successful in anaerobic cultivation of a specific spirochete in association with a small Gram-negative bacillus from the sediment of washings of the buccal cavity of patients ill with mumps. The culture medium used was composed of rabbit serum, 4 parts; extract of the red blood corpuscles of the horse, 1 part; and physiological saline, 5 parts. The spirochete was observed to break up into small granules, in which state it was possible to filter it; but it could not be cultivated from the filtrate unless the original small Gram-negative bacillus were added to it. He was able to produce in the *Macacus sinicus* a parotitis identical with that produced by inoculation of the saliva from the patients, by injecting the cultured spirochetes into the lumen of Stensen's duct or directly into the parenchyma of the gland. He was unable, however, to transmit the disease from monkey to monkey, but reported the production of an orchitis in rabbits which was transmissible in series (6).

A review of the literature shows that the causative agent of mumps has been at various times and by different investigators attributed to bacteria, to a filterable virus, and lately to a spirochete. One gains no conviction however, from a study of the experiments reported up to the present time, that anyone has unquestionably succeeded in inducing mumps experimentally, or has demonstrated the true etiological agent of this disease. There has been a varied choice of experimental animals, of methods of inoculation, and of the character of the supposedly infectious material.

In planning an experimental investigation of mumps we have been

guided by the following assumptions: that monkeys would more likely be a susceptible host than lower animals; that the active agent should come in direct contact with the parenchymal cells of the parotid gland, preferably attended by some injury; and that the causative agent is in the saliva at least in the first stages of the disease.

In order to bring about the most favorable conditions for the experiment it was thought to be advisable to introduce a considerable amount of fresh untreated saliva directly into the parotid glands through the duct of Stensen.

This procedure would of course introduce along with the undiluted hypothetical causative agent a variety of microorganisms, but our experience had indicated that *M. rhesus* has a strong natural resistance to many microorganisms both saprophytic and pathogenic derived from human sources. There would be by this method also an associated injury to presumably susceptible cells. It was expected, since the incubation period of mumps appears to be at least several days, that in the meantime the non-specific microorganisms would disappear. If they should persist we hoped it would be possible to separate the true cause from contaminants by passage in series or by filtration.

By the application of this method of procedure we have been able to obtain from the saliva in a proportion of cases of mumps, in the early stage of the disease, three strains of a filterable virus, free of demonstrable microorganisms even in the first monkey generation, which is uniformly pathogenic for *rhesus* monkeys and induces an acute non-suppurative parotitis which we judge to be mumps. One strain of the virus has been passed in series through seven generations in *M. rhesus* monkeys and has increased in its virulence. We think that the experimental disease can be reproduced at will and indefinitely in series.

Material and Method Used for Inoculation

The original material used for inoculation was fresh saliva obtained by having the patients, with the clinical history, symptoms, and signs of epidemic parotitis, rinse their mouths thoroughly with sterile physiological saline. After disposing of the washings they expectorated for about 2 hours into sterile, wide mouth, glass bottles. During the collection of the saliva the patient was asked to suck the sides of the cheeks in an attempt to obtain as much saliva from the parotid glands as possible.

The first patient from whom saliva was thus obtained was a medical student
who was admitted to Vanderbilt University Hospital about 12 hours after the onset
of slight pain and slight swelling of the gland. He gave a history of contact with a
case of epidemic parotitis 4 weeks previously while spending the Christmas holidays
at his home in Pennsylvania. The saliva was collected over a period of 2 hours at
which time the patient's left gland was quite swollen, and rapidly increasing in
size. His temperature was 103°F. The saliva was immediately used for experimental inoculation after the completion of its collection.

About 2 months later an epidemic of parotitis appeared in the Tennessee Industrial School located at Nashville. Saliva was collected individually from six
of the patients in the same manner as that described above. This material was
iced in an electric refrigerator for a period of about 2 hours after its collection
before being injected into monkeys.

Material from a case in southeast Alabama, where there had been an epidemic
for 3 months, was sent to us at about the same time the epidemic appeared at the
Industrial School. The saliva from Alabama was collected in the same manner as
that of the first case. The bottle was tightly stoppered, sealed with paraffin,
chilled by packing in ice, and mailed to us. The saliva was received the following
day and was used immediately.

Altogether fresh saliva from six cases of mumps has been used for
the inoculation of monkeys. Four of these specimens induced the
specific parotitis, and two of them induced no recognizable clinical
disease. These two specimens were introduced into the same monkey, one in each parotid. This monkey was not killed. Of the four
positive specimens of saliva two were obtained within the first 24
hours after the first noticeable swelling of the parotids of the respective
patients; one was obtained on the 2nd day; the other, from Alabama,
not later than the 2nd day. Of the two negative specimens one was
obtained on the 3rd day of the disease, the other possibly on the 3rd
day, although we are not certain as to the exact time.

These results indicate that saliva taken within the first 24 hours
after the onset of mumps is more likely to be infectious for monkeys
than that recovered at later periods.

The sides of the face of the monkeys were shaved closely so that the changes that
the glands might undergo could be observed easily. The animal was anesthetized
with ether. The orifice of Stensen's duct was exposed by sticking a cotton-covered
forefinger between the lips and down the inside of the cheek, then retracting the
cheek by holding it between the finger and thumb. The duct was cannulated by
inserting a size 22 Luer needle, which had had the point cut away to the shaft producing a blunt end to prevent the penetration of the walls of the duct. It was

found that the procedure of cannulation and injection was more easily performed if a 10 cc. Luer syringe with needle attached and containing the material to be injected were used. The orifice of the duct lies rather deeply and the longer syringe affords a better handle. The usual volume injected into each duct was 2 cc. It is necessary to anesthetize the animal in order to relax the muscles of the cheek sufficiently to permit a free injection. As the injection proceeds under moderate pressure the parotid may be seen and felt as it enlarges, so that one has no doubt about the entrance of the material.

The temperature and the total differential and leucocyte counts were taken before inoculation. The temperature was taken twice daily thereafter; and the total and differential leucocyte counts were made once daily from the day of inoculation until the animal was either killed or completely recovered. In several experiments the temperature and the leucocyte counts were made for a few days before the injection.

Results of Inoculation of Saliva

(a) *Normal Saliva.*—Saliva was collected from two normal individuals by the same method used to collect the saliva from the patients suffering from epidemic parotitis. One of the normal individuals gave no history of mumps, while the other had had the disease during early childhood.

The parotid glands of each of two *Macacus rhesus* monkeys were immediately inoculated by the previously described method of cannulation of Stensen's duct. Each gland of each monkey received 2 cc. of the whole saliva from each of the two individuals. This was the amount of saliva from patients with mumps which was injected into the ducts of each monkey in the reciprocal experiments.

These two monkeys, like those receiving the saliva of patients ill with parotitis, developed an immediate enlargement of the glands which increased in size for 12 to 24 hours and then returned to normal within 72 to 96 hours after the inoculation. They were observed daily for 3 weeks in the same manner as those which received the saliva supposedly containing the virus of epidemic parotitis. Their glands did not show any evidence of enlargement or tenderness after their return to normal. Their temperature showed no secondary elevation and they did not develop a leucopenia.

Ordinarily the parotid glands of a *rhesus* monkey are not at all palpable or barely so; consequently by palpation one can determine quite readily any moderate enlargement.

(b) *Saliva of Patients with Epidemic Parotitis.*—Saliva from Case 1 of mumps was diluted with an equal volume of 0.9 per cent NaCl solution and filtered through a Berkefeld N candle. The filtrate was bacteriologically sterile. 3 cc. of clear fil-

trate were injected into each parotid gland of a monkey through Stensen's duct. After a preliminary swelling which lasted about 48 hours, the glands returned to normal, and remained so. The unfiltered saliva from this patient was very active when injected into monkeys, consequently we believe that the virus in the saliva did not pass the candle, or was so diluted thereby that it was rendered innocuous. Therefore in all other experiments in which saliva from cases of mumps was used, whole fresh saliva was injected. Bilateral injections were made in nearly all instances.

The inoculation of the glands was followed immediately by a definite enlargement due to the volume of the injected fluid. There was a gradual enlargement for 12 to 24 hours, but this was followed by a rapid return to normal in from 48 to 96 hours following the injection. The immediate reaction and the return to normal was similar to that following the injection of normal saliva. The leucocyte count usually rose during the first 24 hours, in some of the cases the number was doubled. This rise was followed by a progressive fall in leucocytes until a definite leucopenic stage was reached which averaged about 4 days after the inoculation. With the development of the leucopenia there was a true and relative monocytosis, a relative lymphocytosis, a marked fall in the polymorphonuclear percentage and total count. In many instances there was a slight rise in temperature on the 3rd to the 5th day following inoculation. This rise in temperature was only of 1 to 2 days duration and the return to normal was usually paralleled by a slight rise in the leucocyte count which was only 1 day in duration in the majority of the cases. (Figs. 1 and 2.)

The above changes were in turn followed by a palpable and visible enlargement and a tenderness of the glands, a rise in rectal temperature, and an edema of the soft tissues over the gland by the 6th to the 8th day after inoculation. If the animal were not killed the edema and enlargement of the glands disappeared after 2 to 4 days. The rise in temperature usually was only 1 to 3 days in duration.

The animals were usually killed the day of or the day following the appearance of edema in order to obtain their parotids for study and for transmission of the virus. They were killed, during ether anesthesia, by exsanguination. The chest was shaved closely, cleansed with alcohol, dried with ether, and then painted with 7 per cent iodine. The heart was located and pierced with a needle, gauge 18, length $3\frac{1}{2}$ inches, attached to a 20 or 30 cc. syringe. As much blood as possible was removed in this manner, and if it were impossible to withdraw sufficient blood to cause death the femoral arteries were dissected and sectioned. The animal after death was placed on one side and the upward cheek shaved, cleaned with alcohol, dried with ether, and painted with 7 per cent iodine. The remainder of the body was covered with lysol-soaked towels. A V-shaped incision was made in the skin over the gland. The point of the V was about 1 cm. from the corner of the lips with the forked ends extending to the neck over the angle of the jaw and the other superiorly to the ear. Care was taken not to include the cheek pouch in the incision. The skin and the fascia were then dissected and held retracted by sterile hemostats. A small piece of the gland was immediately removed, with the ex-

posed gland *in situ*, for bacteriological studies. Aseptic technique was continued as the gland was dissected from its bed and placed in a sterile Petri dish.

CHART 1. Temperature and leucocyte count curves of Monkey 23. It received into each gland 2 cc. of ground emulsion of the left parotid from Monkey 31.

The other gland was removed in the same way. Each of the glands was weighed in a sterile Petri dish and sectioned. Pieces for histological study were fixed in Zenker's solution, 80 per cent alcohol, 10 per cent neutral formalin, and formol-

Zenker's solution. Pieces of the supposedly sterile gland were placed in small bottles containing sterile 50 per cent neutral glycerine. The remainder of the gland was placed in small, sterile, rubber-stoppered bottles. These were placed in an electric refrigerator very near the freezing unit, where the temperature was at about the freezing point.

By the use of the methods just described three strains of the virus we are dealing with were isolated from the saliva of patients from three states of the Union. Bacteriological examination of the parotids inoculated with mumps saliva containing two of these strains, showed no microorganisms; one gland infected with another strain contained a staphylococcus which apparently played no part in the lesion, as determined histologically.

Transmission of Virus from Monkey to Monkey

The material used for passage of the virus through seven generations in monkeys up to date was prepared by taking small pieces of the gland, previously shown to be bacterially sterile, weighing them, and grinding them in a sterile mortar. About 1 gm. of the ground material was then taken up in 10 parts of 0.9 per cent NaCl solution. The heavy material was allowed to settle to the bottom of the tube by allowing the emulsion to sit overnight in the ice box. The emulsion was tested for sterility and if no growth became apparent in the media by the following day, 2 cc. of the turbid emulsion at the top of the tube were injected into each gland of a monkey through Stensen's duct by the same method used in the inoculation of the saliva. (Fig. 3.)

It was found that if the infected glands were allowed to remain in the ice box 2 to 7 days before being ground they were more easily macerated and the emulsion was more uniform in its results, than if the emulsion were made and used immediately after removal of the glands.

As shown in Table I there has been very little or no change in the incubation period, in the temperature curves, and in leucocyte counts in the seven generations of virus; but the most pronounced general reactions of the monkey have been observed in this series. A heightened reaction was noticed in the last three generations when it was observed that at times the animals would be drowsy and listless, and at other times easily excitable and very nervous. The tenderness of the glands was more marked and there was a slight increase in the duration of the edema of the subcutaneous tissues and swelling of the parotids.

TABLE I

Summary of the Transmission of the Virus in Series for Seven Generations

Monkey No.	Generation No.	Return to normal after inoculation (days)	Beginning enlargement (days)	Definite enlargement (days)	Appearance of edema (days)	Duration of edema (days)	Leucocyte response to inoculation — 1st day	Leucocyte response to inoculation — 2nd day	Beginning of leucopenia stage (days)	First rise after leucopenia (days)	Temperature rise after leucopenia (days)	Duration of rise (days)	Temperature rise with symptoms (days)	Duration of fever (days)	Monkey pox	Lymphocytosis
5	1	4	6	6	8	Killed	Rise	Fall	4	6	2	2	8	Killed	True	Relative
6	2	4	5	5	6	Killed	None	None	4	None	2	1	6	Killed	True	Relative
11	3	4	7	7	7	Killed	Fall	Rise	3	4	3	2	6–8	Killed	True	Relative
16	4	3	6	6	6	Killed	Fall	Fall	3	3	3	1	5	2	True	Relative
17	4	4	6	6	6	3	Fall	Rise	6	None	3	1	5	2	True	Relative
21	5	2	4	5	6	3	Fall	Fall	3	5	2–3	2	7	Killed	True	Relative
9	5	2	3	5	7	Killed	Fall	Rise	2	3	3	2	5	Killed	True	Relative
40	6	3	5	6	9	Killed	Fall	Fall	5	5	3	1	6	Killed	—	—
42	7	3	8	8		Killed		Rise	4	5	5	1	8	Killed	—	—
Average..	—	3	6	6	7	3	Fall		3	5	3	1	5–8	2	True	Relative

The figures in the columns refer to the number of days after the inoculation of the glands with exception of the columns referring to the duration of rise in temperature and the duration of edema.

Gross and Microscopic Lesions of the Glands

When the specific experimental parotitis manifests itself by swelling of the glands the subcutaneous tissues and fascia surrounding them usually become quite edematous. The parotids are enlarged both in weight and volume by 2 to 4 times their normal size. They are easily palpable and can be picked up between the fingers; they are boggy and often contain shotty lymph nodes at the lower pole. There is a pitting edema of the overlying cheek. On exposing the glands they are found to be quite congested and through their capsule can be seen small hemorrhagic areas varying in size from pin-point up to 1 to 2 mm. in diameter. The glands as well as the surrounding tissues are very edematous, and there is a weeping of a serous exudate after removal. (Figs. 4 and 5.)

Histologic sections disclose marked destructive changes in the parenchyma of the glands. The parotid of the *rhesus* monkey is a serous gland, and the earliest stage of the lesion in the experimental parotitis is found in acinar cells. The essential lesions are focal in distribution. They are primarily a degeneration and necrosis of a single acinus or small groups of acini. The affected cells become swollen, lose their normal granulation, and become detached. The nuclei show the various changes indicative of necrosis. No nuclear inclusions have been observed, although there are often discrete though faintly staining masses in the cytoplasm in earliest stages. The exact nature of these structures has not yet been determined.

The disintegration of acinar cells takes place before there is a cellular exudate. As the cells die mononuclear phagocytes enter and take up the remains. Later, within the area of necrosis and in the interstitial tissue about many of the ducts, there is an infiltration by lymphocytes in which there are a few eosinophilic leucocytes and plasma cells. Polymorphonuclear neutrophilic leucocytes play no part in the inflammatory reaction. Accompanying the cellular injury there is a diffuse edema of the gland affecting the cells and the interstitial tissues generally. The parenchymal cells become edematous and often elevated from the basement membrane. In the interstitial tissue there is an abundant serofibrinous exudate, and in the neighborhood of the areas of acute parenchymal destruction petechial hemorrhages are

observed. No changes have thus far been found in the submaxillary glands. (Figs. 6 and 7.)

In the parotids of monkeys killed at the height of the disease there are focal areas of parenchymal necrosis of different ages, and we have interpreted this to mean that the changes begin 3 or 4 days after the inoculation, and spread through the gland until there is a sufficient general response to result in enlargement and edema.

Characteristics of the Virus

(a) *Bacteriology.*—The small portions that were removed from each gland *in situ* for cultural study were cut into pieces of about 2 to 3 mm. square. A piece was placed into each of a tube of plain infusion broth, blood broth, and anaerobic beef heart media. In practically every case the gland was found to be bacterially sterile. Noguchi's medium for leptospira was also used for several of the glands but no growth of microorganisms was obtained.

Dark-field examinations of emulsions of the fresh glands made immediately after removal, and also of the emulsion in the transfer of the virus from monkey to monkey, have shown no evidence of spirochetes. Smears, and sections of the glands stained by Levaditi's method for demonstrating spirochetes in tissues, have been entirely negative. Thus cultivation on artificial media and the staining characteristics of the virus still remain unsolved problems.

(b) *Filterability.*—Emulsions of the glands were prepared as for the passage of the virus from monkey to monkey. They were allowed to remain in the ice box for 24 hours, and were then centrifuged at a moderate but sufficient speed for the supernatant fluid to be clear but opalescent. This was decanted and to 10 cc. of the supernatant fluid 0.5 cc. of a 24 hour broth culture of *B. prodigiosus* was added to check the filter. The material was then slowly filtered through a Berkefeld V or N filter at 2 to 5 pounds pressure. Cultures of the filtrate were made and the filtrate stored in the ice box. The cultures being sterile, the filtrate was then inoculated into the glands of the monkeys in the usual volume and by the previously described method for the inoculation of saliva.

In every instance of five trials the filtrate proved to be virulent. The incubation period was from 1 to 4 days longer than for unfiltered material but this was the only change detected in the reaction to the filtered virus.

(c) *Resistance to Freezing.*—The saliva of Case 1, which induced parotitis following its initial injections, was kept in a stoppered bottle in the freezing unit of an electric refrigerator. The saliva was removed from the unit after 3 weeks and allowed to melt. Another monkey was inoculated with it in the same manner as the first. Its reaction was identical with that of the first monkey with the exception that the incubation period was shorter by 1 day.

(d) *Resistance to Drying.*—Pieces of the glands of the monkey receiving the fifth transfer were placed in a Petri dish immediately after removal. The Petri dish was packed in carbon dioxide ice and allowed to remain for 30 minutes. Com-

letely frozen, it was transferred to a desiccator in which there was phosphorus
entoxide.   The desiccator had been packed in ice and was connected to a vacuum
ump.   The pressure was then reduced to 2 mm. of mercury.   The ice-packed

DATE	3-22	3-23	3-24	3-25	3-26	3-27	3-28	3-29	3-30	3-31	4-1	4-2
TOTAL P.M.N.	6,786	11,762	4,922	5,341		1,740	1,680	1,219	3,525	3,108		5,580
TOTAL LYM.	4,563	4,251	5,457	5,123		3,712	3,840	3,604	3,525	4,704		6,200
TOTAL MONO	351	327	321	436		348	480	477	450	588		670
	GLANDS INOCULATED			NORMAL		SLIGHT ENLARGEMENT	ENLARGING TENDER	EDEMA APPEARING	EDEMA MARKED	EDEMA AND SIZE DECREASING		ALMOST NORMAL
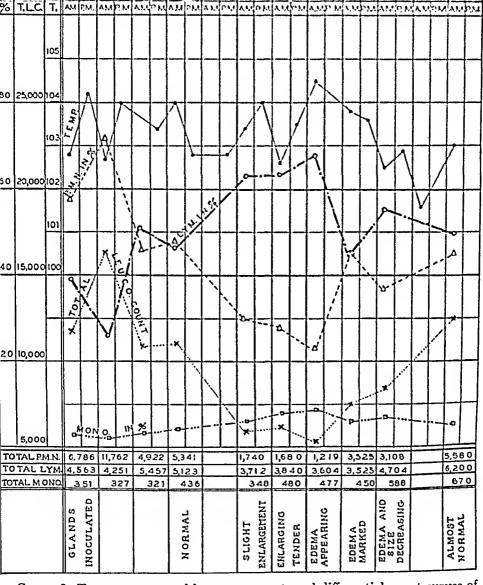
CHART 2. Temperature, total leucocyte count, and differential count curves of
Monkey 20.   The base numbers refer to the total polymorphonuclear neutrophils,
lymphocytes, and monocytes.   Each gland was inoculated through Stensen's
duct with 2 cc. of a filtered emulsion of the gland of the fourth generation monkey.

desiccator was placed in the ice box and allowed to remain there for 4 days. The dried pieces of the glands were removed and placed in small sterile ampules and sealed.
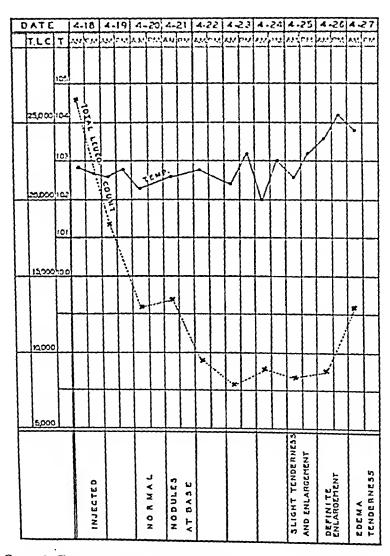
CHART 3. Temperature and total leucocyte count curves of Monkey 30, sixth generation. It was inoculated by the routine method described in the text with bacterially sterile filtrate of the emulsion of Monkey 19 which had in turn been infected with the same inoculum as Monkey 20 (Chart 2).

TABLE II

Summary of the Findings of the Filtrate Experiments

Monkey No.	Return to normal after inoculation	Beginning enlargement	Definite enlargement	Appearance of edema	Duration of edema	Leucocyte response to inoculation 1st day	2nd day	Beginning of leucopenia stage	First rise after leucopenia	Temperature rise after leucopenia	Duration of rise	Temperature rise with symptoms	Duration of fever	Monocytosis	Lymphocytosis
	days	*days*	*days*	*days*	*days*			*days*	*days*	*days*	*days*	*days*	*days*		
15	3	5	7	8	Killed	Fall	Rise	3	6	5	1	8	Killed	True	Relative
19	2	5	6	8	Killed	Fall	Fall	3	5	5	1	8	Killed	True	Relative
20	3	5	6	7	3	Rise	Fall	3-5	4	5	1	7	2	True	Relative
28	3	6	7	8	Killed	Rise	Level for 3	5-7	6	5	2	7	2	—	—
30	2	7	8	9	Killed	Fall	Fall	4	None	5	2	8	2	—	—
33	4	7	9	10	Killed	Rise	Fall	6	8	6	2	10	1	—	—
Average..	3	6	7	8	3	Rise–fall	Fall	3-5	4-6	5	1-2	8	1-2	True	Relative

The figures in the columns refer to days after inoculation like those of Table I.

The dried gland remained in the ampules for 7 weeks. An ampule was then opened and the contents emulsified for inoculation into a monkey. The emulsion was prepared much in the same way as that for the transfer of the virus in series, by grinding the dried material in a proportionate volume of 0.9 per cent saline solution. The animal developed the typical leucocyte response, rise in temperature, enlargement of the glands, and edema of the subcutaneous tissues in the usual sequence.

(c) *Resistance to Glycerination.*—Small pieces of the same gland that was frozen and dried were also placed at the same time in 50 per cent neutral glycerine. After being stored in the ice box at about 10°C. for 7 weeks an emulsion of the pieces was prepared. A monkey inoculated with the usual volume of the emulsion developed the typical clinical response previously mentioned after the usual incubation period, showing that there had been no loss in the virulence of the virus.

Immunity

(a) *Reinoculation Experiments.*—Two of the monkeys which were allowed to overcome their infection for clinical observations were again inoculated, 2 months after recovery. An emulsion of the glands of the monkey of the sixth transfer was prepared by the previously described method. Each parotid of the two monkeys previously ill with the malady received 2 cc. of the emulsion. A normal monkey was given 2 cc. of the emulsion into the left parotid as a control.

The two monkeys which had previously been inoculated and had developed swelling and tenderness of the parotids, a rise in temperature, and a leucopenia, failed to show any symptoms of infection following reinoculation. These two monkeys were observed for 3 weeks after the inoculation.

The normal monkey which received the emulsion in only the left gland developed a slight rise in temperature, a typical enlargement of the inoculated parotid on the 7th day after the inoculation, and 4 days after its initial return to normal size. Edema of the subcutaneous tissues appeared on the 8th day after inoculation, and the animal was killed on the 9th day after the temperature had reached its peak and had begun to recede. The gross and microscopic changes were characteristic of the experimental disease.

(b) *Attempt to Neutralize the Virus with Sera of Patients Recently Recovered from Mumps.*—An attempt was made to determine whether or not the sera from patients recently recovered from mumps would have an inhibiting effect upon the virus inoculated into monkeys. In pursuance of this object two sets of experiments were made.

In the first set the serum from an adult human donor who seemed to have a reliable history of never having had an attack of mumps was used as a control. Sera from two patients who had recently recovered from mumps—about 2 months previously—were used to test their possible antiviral effect. Four monkeys were used. Into one gland of each monkey a mixture of equal parts of virus emulsion and "normal" serum was injected. Into the opposite parotid of each a similar mixture of virus and "immune" serum, from two recently recovered patients, was injected at the same time.

The virus suspension was made by emulsifying an infected gland in 0.9 per cent saline solution in a proportion of 1 to 10, just as the inoculum in other experiments had been prepared. The mixtures of serum and virus were incubated at 37°C. for 2 hours, and were then injected into the parotids.

The results were quantitatively inconclusive as to the antiviral effect of the immune serum. Three of the parotids receiving normal serum and virus became swollen and edematous in due time; the fourth showed no recognizable change. Of the four glands which received immune serum, one became swollen and edematous 2 days later than the opposite, control side; one gland became slightly enlarged, without facial edema; the remaining two showed no change. One of the monkeys, however, showed no enlargement of either parotid.

In a second set of experiments an attempt was made to compare the effect of immune serum upon the usual virus emulsion, and a sample of the same diluted to half its strength. Each inoculation was made into a separate monkey. As a control the serum from a supposedly normal placental blood was used. Two pairs of monkeys were inoculated. One monkey received a mixture of full strength virus emulsion plus an equal quantity of normal serum; another received half strength virus emulsion plus an equal volume of normal serum. In the second pair immune serum and virus emulsion were prepared and inoculated in the same way. The serum-virus mixtures were kept in a refrigerator just below the freezing point for 24 hours before the inoculation. They were not incubated at 37°C.

Each of the two monkeys which received normal serum and virus developed in due course swelling of the parotids and facial edema. The monkey which received diluted virus plus immune serum remained normal, at no time showing a swelling of the gland. The one which received strong virus and immune serum developed an erythematous rash on the 14th day associated with a swelling of the parotids, and this condition, not observed by us before, persisted for several days. The parotids were distinctly enlarged but there remained the question in our minds whether or not the skin eruption associated with edema was a manifestation of serum sickness which involved the parotids in the inflammatory reaction as well.

While this problem requires much more extensive investigation, we may say that none of the parotids inoculated with virus plus immune serum presented so marked an enlargement as the corresponding control, and where both serum-virus mixtures were inoculated into the

same animal the enlargement of the immune side was delayed 2 to 3 days. In two of the experiments there was no response from the glands receiving immune serum plus virus while the corresponding controls were positive in the usual manner.

We judge from these few preliminary experiments that there is some inhibiting effect of serum from patients recently recovered from mumps upon the virus with which we are working.

DISCUSSION

The infectious agent which we have obtained from the saliva of patients in the early stages of mumps presents characteristics of a filterable, cytotropic virus having a predilection for the parenchymal cells of the parotid glands of *rhesus* monkeys. The virus is free of demonstrable microorganisms; it is filterable and resistant to drying and glycerination; it causes a lesion which is primarily a degeneration and necrosis of the parenchymal cells of the parotid; and it confers immunity to reinoculation. There are no visible structures associated with it which we can interpret as microorganismal. No spirochetes have been observed in smears, in dark-field preparations of fresh lesions, or in sections of tissue stained by Levaditi's method.

There is good evidence, we consider, that this virus is the cause of mumps. We have not found a similar virus in saliva other than that of patients suffering from mumps, although we have not yet tested a large number of specimens. The virus has been demonstrated in four out of six specimens of saliva from cases of mumps.

The clinical disease induced in *M. rhesus* monkeys, whose parotids have been inoculated with the virus, is analogous to mumps in the human being. The histology of the parotitis of human mumps is as yet undetermined, but the lesions of the experimental disease are quite comparable to those found in the specific orchitis of mumps, except that in the latter there is a considerable neutrophilic leucocytic response. While our experiments attempting to neutralize the virus with serum of mumps-immune patients are inconclusive, they indicate so far as they go, that such sera have an inhibiting effect upon the virus.

It is well known that the saliva of a small proportion of people may harbor the virus of herpes simplex. The possibility that we are deal-

ing with the herpetic virus, we think is ruled out by the fact that cutaneous, corneal, and intracerebral inoculations of rabbits with the experimental virus is innocuous so far as we have determined. There are likewise no nuclear inclusions in the experimental lesion. The insusceptibility of rabbits to the virus would also eliminate an accidental vaccinal contaminant. This is further obviated by the fact that monkeys immune to the experimental disease under consideration are susceptible to the vaccine virus. We are not aware of other known viruses with which the virus we have demonstrated may be confused.

Our judgment that we are dealing with a filterable cytotropic virus which is the true cause of mumps is based upon the above mentioned data. Further experiments are being conducted, however, to elucidate more clearly the characteristics of the virus.

CONCLUSIONS

1. From four out of six specimens of saliva from six cases of mumps in the early stages of the disease, a filterable cytotropic virus has been obtained which induces in *M. rhesus* monkeys, following inoculation of the parotid glands through Stensen's duct, an acute, non-suppurative parotitis analogous to mumps.

2. This virus has not been found in normal saliva, nor does it correspond to any known virus with which we are familiar.

3. The virus is free of demonstrable microorganisms including spirochetes.

4. It is judged that this virus is the causative agent of mumps.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Monkey 31. Photograph taken on the 6th day following inoculation of saliva from two patients ill with epidemic parotitis. The left gland, the larger of the two, received 2 cc. of saliva from a patient during the 1st day of illness. The right gland, which is only moderately swollen, received 2 cc. of saliva from a patient during the 2nd day of illness.

FIG. 2. The left side of the monkey shown in Fig. 1. Note the pitting of the edematous tissues over the swollen gland which resulted from pressure.

PLATE 2

FIG. 3. Monkey 21. On the 7th day after inoculation and on the 2nd day of marked swelling. There was marked bilateral enlargement of the parotids and edema of overlying tissues. It received into each duct 2 cc. of the emulsion of the gland used for the fifth transfer.

FIG. 4. One of the experimentally infected glands *in situ* showing the hemorrhagic areas and marked congestion as described in the text.

FIG. 5. The gland of Monkey 42, the seventh generation, showing the marked difference in size of a normal, non-inoculated gland and a diseased gland of the same monkey. The diseased gland was 2½ times as heavy as the smaller normal gland which showed no evidence of being infected either in the gross or by histological study. Actual size.

PLATE 3

FIG. 6. Photomicrograph of section of the diseased gland in Fig. 5 showing an infected lobule in which there are focal areas of necrosis with cellular infiltration. The interstitial edema is also apparent. × 80.

FIG. 7. A focal area of necrosis in which there has been a moderate infiltration by large mononuclear phagocytic cells and lymphocytes. × 450.
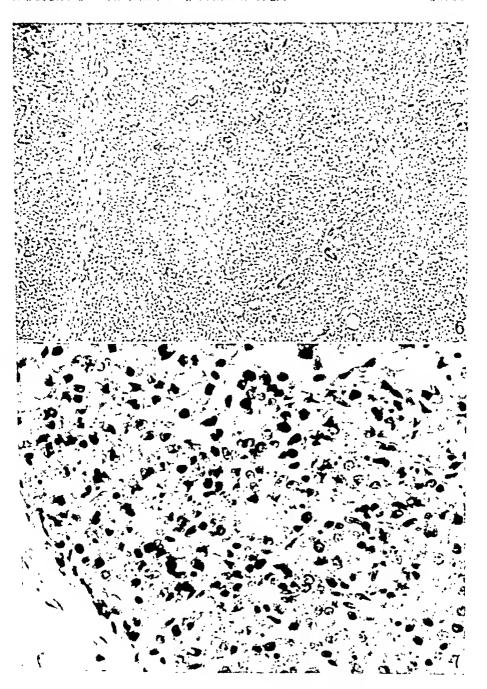
(Johnson and Goodpasture: Etiology of mumps)

5

STUDIES ON THE NERVOUS SYSTEM IN DEFICIENCY DISEASES

II. Lesions Produced in the Dog by Diets Lacking the Water-Soluble, Heat-Stable Vitamin B₂(G)[*]

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PLATES 4 TO 6

It is now generally conceded that black tongue in dogs as produced by Goldberger and his coworkers (1) with diets lacking the water-soluble, thermostable vitamin B₂(G)[1] has many features in common with the disease known as pellagra in man. These features are the cutaneous, lingual and buccal lesions, the diarrhea and the salivation. Another frequent manifestation of pellagra in man, however, is the involvement of the nervous system as described by Sandwith (2), Singer and Pollack (3), Winkelman (4) and Pentschew (5), who found degeneration of the medullary sheaths of the posterior columns and frequently also of the lateral and anterior pyramidal tracts of the spinal cord. Lesions of the nervous system have not as yet been described in dogs with black tongue, and it therefore seemed worth while to investigate the possibility of their presence in animals subjected to a well controlled dietary régime.

[1] The physiological properties of what was formerly called vitamin B have since been shown to be due to at least two substances, a heat-labile, antineuritic factor and a thermostable, growth-promoting substance which appears to prevent pellagra. These factors have been designated vitamins B and G by some investigators and B₁ and B₂ by others. The latter nomenclature is employed in this paper. The term vitamin B complex refers to the undifferentiated mixture as it occurs in nature.

It was more than tacitly assumed by Goldberger and his associates that the essential factor lacking in the diet they employed was the pellagra-preventive (P-P) substance or vitamin B_2. The faults of the regimen make it at once apparent that the effects produced on the animal may be multiple rather than single in nature. The diet is inadequate in salts. It is recognized to be low in protein, and the level of casein in the ration does not preclude the possibility of a cystine deficiency. The relatively poorer biological value of the maize proteins becomes of importance in this problem. Goldberger recognized this when, in his work done prior to the discovery of vitamin B_2, he suggested that an amino acid deficiency is involved in the etiology of pellagra. Furthermore, the greater part of the diet is composed of natural foods, and the antineuritic substance is supplied by them. It is not impossible that the amount of vitamin B_1 contained in the ration is subminimal and that this is of importance in producing certain of the features of the syndrome in question. As this vitamin is not found in nature entirely separate from the so called pellagra-preventive substance, the diet contains a small amount of vitamin B_2. How great the influence of this slight but constant intake is in delaying the symptoms directly caused by deprivation of the B_2 factor cannot be measured. Moreover, the inclusion in the ration of so much cornmeal exposes it at once to the criticism of those who believe that pellagra is caused by the ingestion of maize (6); and although zeism is now regarded by most of the workers in this field as a defective theory, it has yet to be entirely disproved.

To obviate the apparent faults of such a diet the aid of Dr. George R. Cowgill of the Department of Physiological Chemistry was enlisted in the preparation of the artificial ration employed in the experiment forming the basis of the present communication. This diet is much more highly purified with respect to the water-soluble vitamin B_2 than any ration hitherto employed in these laboratories for feeding experiments with dogs.

In an earlier contribution on the changes associated with deprivation of the vitamin B complex (7) it was found that one animal, which had subsisted on a diet deficient in both vitamins B_1 and B_2, showed degeneration of the median dorsal fasciculi of the spinal cord. The present study is an outgrowth of this earlier investigation.

Experimental Procedure

A preliminary training of at least 2 weeks was allowed for each of the eight dogs used in this study in order to ascertain whether the animal was suitable for the object of the experiment. During this period an excessive amount of whole yeast (2 gm. per kilo) was given daily to insure a uniform nutritional history with respect to the vitamin B factors (B_1 and B_2).

Diets Employed.—At the beginning of the experiment the animals were fed the following ration:

TABLE I

Constituents	Per cent
Casein-Harris* (87.1 per cent protein)	28.0
Sucrose	31.0
Lard	30.0
Butter fat	6.0
Salt mixture†	1.8
Bone ash	3.2

* Furnished by the Harris Laboratories, Tuckahoe.
† Karr-Cowgill (8) salt mixture.

This diet formed the basal ration for about 5 months from the beginning of the experiment. At that time the question arose of whether the large proportion of fat in the diet was exerting a sparing action on vitamin B_2 similar to that believed by Evans and Lepkovsky (9) to be true for the antineuritic vitamin B_1. Five of the remaining seven animals were, accordingly, given a diet in which the proportion of the proximate principles was changed to yield a ration poor in fat, as follows:

TABLE II

Constituents	Per cent
Casein-Harris (87.1 per cent protein)	22.0
Sucrose	35.0
Rice starch	29.0
Butter fat	10.0
Karr-Cowgill salt mixture	1.4
Bone ash	2.6

Dogs 3 and 4 were allowed to act as controls and continued to subsist on the former diet (Table I). The fat-poor ration (Table II) was mixed with a known quantity of water and offered as a gruel at first but was later baked into a biscuit

It is to be noted that the protein level in calories per 100 gm. of diet is identical in both rations (Table III).

TABLE III

Diet	Distribution of calories			Calories per gm. of diet
	Protein	Fat	Carbohydrates	
I	18	59	23	5.5
II	18	21	61	4.2

Antineuritic vitamin B_1 was administered separately in the form of a concentrate prepared from rice polishings by Block (10) after tests with rats showed an absence of the heat-stable vitamin B_2 from this product. The concentrate was injected intravenously or subcutaneously at such times when oral administration (by stomach sound) was ineffectual owing to vomiting or diarrhea. The material, assayed by the pigeon method, was administered in excess of the minimal requirements of the dog as established by Cowgill (11). A record of the amount of food consumed daily was kept throughout the experiment as a gauge of the appetite and indication for the administration of the antineuritic vitamin concentrate.

Material Studied and Technic Employed.—Complete necropsies were performed on all the animals and the thoracic and abdominal organs were examined grossly and, where indicated, also microscopically. Blocks of these viscera were fixed in a solution of formaldehyde, u.s.p. (1:10), embedded in paraffin, and stained with hematoxylin-eosin. The findings other than those in the nervous system, however, will be mentioned only briefly in this communication.

Blocks of the brains and spinal cords, immediately upon removal from the animals, were fixed in 95 per cent alcohol, in the diluted solution of formaldehyde and in Müller's solution. In the latter two fixatives were placed pieces of the brachial plexuses, sciatic, vagus and phrenic nerves. The alcohol-fixed material was embedded in celloidin and stained with toluidine blue. The formaldehyde-fixed material was used in part for the demonstration of fat by the Sudan III method, myelin sheaths by the Spielmeyer and axis cylinders by the Bielschowsky methods; in part it was employed for the demonstration of medullary sheaths by the Kulschitzky method after mordanting in Weigert's rapid mordant. The material fixed in Müller's solution (without formaldehyde) was stained with osmic acid, embedded rapidly in celloidin and sectioned at 30 microns. Blocks of the spinal cords were sectioned longitudinally as well as transversely, and an average of eight blocks at different levels were taken from each cord. Special effort was made to include the spinal nerve roots in the microscopic preparations.

RESULTS

Dog 1.—A fat female, weighing 9.4 kilos, was given enough food daily to maintain a weight of 7.0 kilos. Lost appetite on 21st day and was given antineuritic

vitamin concentrate intravenously. Intraperitoneal injection of this product on 36th day followed by intravenous injection on 41st day. Animal found dead at 9:00 a.m. of 43rd day and necropsy performed at 2:00 p.m. Final weight: 8.5 kilos.

A large intra-abdominal hemorrhage was found originating from a granular, friable mass attached to the peritoneum in the region of the xiphoid. Microscopically this mass was composed of granulation tissue in which there was much necrosis, hemorrhage and leucocytic infiltration. It represented a localized peritoneal reaction, the result of the injection of the vitamin concentrate.

In the gross, no lesions were found in either the central or peripheral nervous systems in this animal or in the other seven. Microscopic examination of the entire nervous system revealed a normal picture. Particularly was there no evidence of degeneration found in the medullary sheaths of the peripheral nerves and spinal cord.

Dog 2.—Female, weighing 5.4 kilos, was given enough food to maintain that weight. Ate fairly well throughout whole experimental period. Animal operated on for strangulated hernia on 51st day and in excellent condition by 54th day. On 140th day animal was muzzled on suspicion of coprophagy. Change in diet made on 168th day. Vomiting of undigested food quite frequent in last 2 months of régime. Death occurred suddenly on 214th day, without any terminal symptoms. Final weight: 4.2 kilos.

Necropsy was performed about 2 hours post mortem. There was no evidence of dermatitis, gingivitis or glossitis. The entire gastrointestinal tract was lined by an intact mucosa.

In the Marchi preparations of the sciatic nerves and brachial plexuses a moderate degree of degeneration of the medullary sheaths was seen. Somewhat less degeneration was found in the phrenic and vagus nerves. Fat could not, however, be demonstrated with Sudan III in any of these nerves. The spinal cord revealed no change by any of the staining methods employed, and this was also true of the cerebrum.

Dog 3.—Female weighing 4.3 kilos. Ate fairly well throughout whole experimental period. After 98th day vomiting of partly or wholly undigested food, and intermittent diarrhea. On 154th day animal was muzzled on suspicion of coprophagy. Death occurred suddenly on 218th day. No terminal symptoms were manifested. Final weight: 3.2 kilos.

Examination was made approximately 5 hours post mortem, at which time the animal was found to be in a good state of nutrition. There was no evidence of dermatitis, gingivitis or glossitis. The teeth were in good condition. The entire gastrointestinal tract presented a normal picture.

All the peripheral nerves showed a marked degree of degeneration of the medullary sheaths in the Marchi preparations, and a similar change was present in the posterior nerve roots of the spinal cord. In spite of these extensive changes in the Marchi preparations, Sudan III stains revealed but a slight amount of fatty

change.   In Spielmeyer preparations only the posterior nerve roots showed definite degenerative changes.

In the spinal cord a few scattered black droplets were demonstrable by the Marchi method.   These droplets were present in greatest abundance in the posterior fasciculi and to a less extent also in the anterior cortico-spinal and the dorsal and ventral spinocerebellar tracts.   Demyelination of these tracts could not be demonstrated by any of the other staining methods.   Nissl preparations of the spinal cord revealed no changes in the white matter, but many of the nerve cells of the anterior horns were shrunken and hyperchromatic and contained small, dark pericellular incrustations.

Kulschitzky, Marchi, Sudan III and Nissl preparations of the cerebrum, brain stem and cerebellum were all essentially negative.

Dog 4.—Male weighing 7.4 kilos.   Ate fairly well throughout whole experimental period.   Intermittent diarrhea after 100th day.   Vomiting of undigested food quite frequent during last 2 months of régime.   On 224th day animal was apathetic and walked with an unsteady gait.   Death occurred suddenly on 228th day.   Final weight: 4.0 kilos.

Necropsy was performed about 6 hours post mortem.   The animal showed marked evidence of loss of subcutaneous fat but no dermatitis.   There were no lesions on the tongue, gingival or buccal and pharyngeal mucous membranes.   The heart was dilated but showed no other change.   The gastric mucosa was intact, but the duodenal mucosa contained three superficial ulcers each measuring 4 mm. in diameter.   The remainder of the intestinal tract was lined by an intact mucosa.

Marchi preparations of the brachial plexuses and the vagus and sciatic nerves showed a striking degree of degeneration of the medullary sheaths; these observations were amply confirmed by both the Spielmeyer and Kulschitzky methods.   In the Sudan III preparations the degenerative changes appeared distinctly to be less severe.   The posterior and anterior nerve roots of the spinal cord showed marked degenerative changes in the Marchi preparations.   In the spinal cord itself a distinct, paramedian, wedge-shaped zone of degeneration was present in the fasciculi graciles.   Nissl preparations of the cord revealed a glia proliferative response in the posterior columns corresponding in size and shape to the lesion seen in the Marchi preparations.   The glia partaking in this reaction were the rod-shaped Hortega cells, fat granule cells and fibrous astrocytes.   Sections stained by the Kulschitzky and Sudan III methods failed to reveal fatty changes.   The degenerative lesion could be traced through all levels of the spinal cord.   Neither in the cells of the anterior horns of the cord nor in those of the motor cortex of the cerebrum were any changes found in the Nissl preparations.

Dog 5.—Male weighing 4.8 kilos.   Given fat-poor diet beginning 109th day. Ate fairly well first 165 days of experiment; thereafter incomplete food intake with 20 per cent loss of body weight during subsequent interval of 54 days.   Small ulcerative lesions on legs and scaly skin shortly before death on 220th day of experiment.   Final weight: 3.7 kilos.

The animal was necropsied immediately after death. Over the bony protuberances of the pelvis and the fore legs the skin was excoriated, apparently from contact with the cage floor. Little adipose tissue was present subcutaneously. There was no sign of gingivitis or glossitis, and the entire gastrointestinal tract was free of ulceration.

The sciatic, vagus and phrenic nerves as well as the brachial plexuses showed by the Marchi method outspoken degeneration of the myelin sheaths. Sudan III preparations of the same nerves showed only occasional fat droplets within the endoneurium of each fasciculus, but the Spielmeyer preparations revealed swollen medullary sheaths and balls and balloons of degenerated myelin.

The posterior nerve roots of the spinal cord, and to a less extent the anterior nerve roots also, showed degenerative changes in the sections prepared by the Marchi method (Fig. 4). The posterior columns of the spinal cord, particularly the columns of Goll, were markedly degenerated at all levels. A diffuse cellular gliosis and several glia rosettes were seen in the columns of Goll in the preparations stained by the Nissl method. The gliosis had a triangular shape with the apex pointing toward the central canal of the spinal cord. The motor horn cells were somewhat hyperchromatic but were otherwise not altered.

An occasional nerve cell in the cerebral cortex had foamy, honeycombed cytoplasm, but the giant motor cells of the precentral convolutions were normal.

Dog 6.—Female, weighing 6.7 kilos, was given enough food calculated to maintain a weight of 6.0 kilos. Ate fairly well for 7 months, then incomplete food intake for last 85 days of experiment. Dog muzzled on 147th day on suspicion of coprophagy. Change in diet made on 172nd day. Diarrhea and vomiting of partly or wholly undigested food occurred on 160th day and persisted intermittently through remainder of experimental period. Salivation noted on 297th day and became more profuse subsequently. Breath foul; gums and cheeks pale. Death on 300th day. Final weight: 3.0 kilos.

Necropsy was performed immediately after death. The animal was emaciated, and there was loss of hair over the back. Ulcerations, 1 to 2 cm. in diameter, were present on the lateral surfaces of the fore and hind limbs. The buccal mucous membranes were strikingly pale but intact. There were no lesions on the tongue or gums. The mucosa of the whole gastrointestinal tract was intact but microscopically contained numerous cells undergoing mitotic division.

A marked degree of degeneration of the medullary sheaths was present in the brachial plexuses, the sciatic and vagus nerves. Both phrenics were also demyelinated, but to a milder degree. This degenerative lesion was seen in the Marchi, Sudan III, Spielmeyer and Kulschitzky preparations with equal clarity (Figs. 1, 2 and 3). That it was of long standing was apparent from the fact that fat-containing phagocytes were in evidence in the interstitial connective tissue; the axis cylinders, nevertheless, were in a good state of preservation.

The picture in the spinal cord was exceedingly striking as regards degeneration in both the posterior nerve roots and the posterior columns. In contrast to the

severe involvement of the posterior roots the anterior nerve roots were only slightly implicated. Degeneration of the posterior columns (Fig. 5) was found in all parts of the spinal cord. The funiculi graciles were completely destroyed and, to a less extent, the funiculi cuneati were also involved. In the latter columns, the medial halves were less severely injured than the lateral halves. Lissauer's fasciculi were completely degenerated, and the same was true of the central parts of the posterior nerve roots which radiate into the posterior horns. Rare, isolated fibers undergoing degenerative changes were found in nearly all the other fiber tracts of the spinal cord, sensory as well as motor. In addition to these lesions, the proximal end of the seventh cranial nerve was found to be almost completely degenerated.

Nissl preparations of the spinal cord showed proliferation of the protoplasmic glia and the microglia phagocytes in the posterior columns. The nerve cells in the gray matter of the cord, including Clarke's column, revealed no changes, nor were lesions found in the cortical gray of the cerebrum.

Dog 7.—Female weighing 4.3 kilos. Ate fairly well for 7 months, thereafter incomplete food intake resulting in weight loss to 2 kilos. Animal observed eating stools on 125th day; subsequently muzzled. Given fat-poor diet after 168th day. Vomiting and diarrhea after 150th day occurring intermittently until death on 331st day. Final weight 2.0 kilos.

Post mortem examination was performed immediately after death. A purulent conjunctivitis was present in both eyes, but there was no trace of cataract formation. The buccal mucous membranes were red and in spots superficially ulcerated. There was ulceration and bleeding of the gum over the upper left canine tooth. The tongue was normal. The gastrointestinal mucosa was intact grossly and microscopically contained numerous mitotic figures. Both parietal and chief cells were present in great numbers in the gastric mucosa.

An extreme degree of degeneration of the medullary sheaths was found in the peripheral nerves. Much phagocytosis of fat on the part of large mononuclear cells could be seen in the interstitial connective tissue. Many medullated fibers were left completely unstained in the Spielmeyer and Kulschitzky preparations as a consequence of their complete degeneration. In the vagus nerve, evidence of degeneration was seen only in the Marchi preparations.

Marked degenerative changes were present in the posterior nerve roots of the spinal cord, but the anterior roots were completely spared. The posterior columns, from the lumbar to the cervical region, were completely degenerated, but the columns of Goll suffered more than those of Burdach. The fibers that pass from the posterior columns into the posterior horns likewise displayed a marked degree of degeneration (Fig. 6). No other fiber tracts than those already mentioned were in any way implicated in the demyelinating process. An illuminating picture of the degeneration in the posterior columns was seen in the longitudinal sections of the cord stained by both the Marchi and Kulschitzky methods (Fig. 7). In spite of the severe changes in the medullary sheaths, the Bielschowsky preparations revealed normal axis cylinders.

Astrocytes and phagocytic glia were found in greatly increased numbers in the posterior columns of the spinal cord stained by the Nissl method. The nerve cells of both the cord and cerebrum presented a normal morphology.

Dog 8.—Female weighing 4.4 kilos. After 122nd day given the fat-poor diet. Ate fairly well for first 235 days, up to which time original weight (4.2 kilos) was maintained. Incomplete food intake during last 2 months resulting in loss of 25 per cent of body weight. (Antineuritic vitamin concentrate was not available during this period.) Coprophagy noticed on 98th day and dog consequently muzzled. Diarrhea and vomiting of partly or wholly undigested food observed occasionally. Death without terminal symptoms occurred on 289th day. Final weight: 3.1 kilos.

This animal was necropsied 3 hours post mortem. The skin was excoriated over the joints of the fore legs. The buccal mucous membranes, the tongue and the gums were in good condition. There were no ulcers in the gastrointestinal tract. The heart was dilated and the left ventricle was hypertrophied. Microscopically the myocardium presented large zones of necrosis that were undergoing organization. The coronary arteries and arterioles showed no evidence of organic occlusion to account for these infarct-like lesions.

The vagus and sciatic nerves revealed striking degenerative lesions of the medullary sheaths. Surprisingly enough, however, the brachial plexuses showed only slight evidence of injury, and the phrenics appeared to be entirely normal.

A slight degree of degeneration was present in the posterior nerve roots of the lumbar region of the spinal cord; at other levels the posterior roots were normal. The anterior roots were uninjured at all levels. Within the cord, the columns of Goll alone were partially destroyed, but even here the axis cylinders were well preserved. Longitudinal sections of the spinal cord stained by the Marchi method confirmed the fact that only the funiculi graciles were injured.

In Nissl preparations of the cord, a feeble proliferative response on the part of the glia was seen in the columns of Goll. No neuronal lesions were found in either the cord or cerebrum.

DISCUSSION

The present investigation had been in progress over 5 months when it became apparent that the disease, black tongue, was not developing with the vitamin B_2-deficient diet employed. It was at that time that the possible sparing action of fat on this vitamin came under consideration, as already mentioned, and the change was made to the relatively fat-poor diet. In spite of this change the rate and course of the development of the disease complex was apparently unaltered, for the animals only very gradually, though progressively, lost weight and developed persistent vomiting, diarrhea and marked muscular

weakness.   Moreover, the reduction in dietary fat did not shorten the period of survival, as the two animals (Nos. 3 and 4) which continued to subsist on the fat-rich ration died long before three of the others. The oral lesions characteristic of black tongue were lacking in all animals; only one had slight ulcers on the buccal mucous membranes, and one other salivated.   In addition, the customary rapidity of onset and progression of black tongue stood out in sharp contrast to the slowly developing disease produced by the artificial ration lacking only vitamin B_2.

From these observations alone it seemed apparent that something more than a deficiency in vitamin B_2 is responsible for part of the picture of black tongue.   This disease may be produced by a multiple deficiency, and in that respect approximates pellagra more closely than the disease produced by a well balanced artificial ration lacking one factor only.   It is not denied that the diet employed by Goldberger and his associates was deficient in vitamin B_2; it is simply affirmed that no one part of the complex picture they produced could with certainty be attributed to any one specific factor.   It is also affirmed that the artificial ration employed in the present study permits the conclusion that whatever disease picture is produced is due to a lack of what at present is regarded as a single factor; namely, vitamin B_2.

A word more needs to be said concerning the diets employed in the present investigation.   They were fed to the animals in quantities calculated to be sufficient to maintain body weight.   Their known caloric content of protein, carbohydrate and fat permitted a proper balance of these substances to meet the requirements of adult dogs. Yet after prolonged intervals the animals invariably lost weight, which was due in whole or in part to vomiting or diarrhea.   To be sure these disturbances were probably the result of the vitamin deficiency, but is it not possible that the resultant loss of such a substance as protein, for example, was responsible for the anatomic changes produced in the nervous system?   A partial answer to this question is already available (7).   Dogs that were deprived practically completely of protein, carbohydrate and fat failed to show any lesions in the spinal cords, although they did have some degeneration of the medullary sheaths of the peripheral nerves.   However, that condition represented an

experiment of short duration, and is therefore not comparable to the present long one. Experiments are now in progress in this laboratory that have been devised to yield a long standing protein deficiency in the hope that the question of its possible effect on the nervous system will be answered.

The changes in the spinal cord observed in all the animals of this experiment except Dogs 1 and 2, which died before any of the others, consisted of degeneration of the posterior columns, particularly those of Goll, degeneration of the posterior nerve roots and, in two animals, of slight degenerative changes in most of the other fiber tracts. The degenerated medullary sheaths were being replaced by gliosis. Changes of this kind, as already mentioned, have been described in pellagra. They are not found, of course, in every case of this disease, but occur with sufficient frequency to be considered an integral part of it. Another very frequent lesion in pellagra is the axonal change in the nerve cells of the motor horns and precentral gyri, which change, however, was entirely absent in these animals.

With regard to the degenerative changes in the peripheral nerves of the dogs in this experiment, it must be stated that Righetti (12) alone has ever presented anatomic evidence of polyneuritis in a case of pellagra. What the incidence of this condition in pellagra really is cannot be stated with any degree of accuracy, inasmuch as the peripheral nerves have been examined apparently but rarely at necropsy. Pellagra being a multiple deficiency disease, it is possible to attribute polyneuritis to absence of the antineuritic vitamin B_1. In this investigation, however, repeated intravenous or subcutaneous administration of this vitamin adequately supplied the animals' needs as far as could be determined; yet there was present degeneration of the medullary sheaths of the peripheral nerves of generally increasing severity with the length of time the animal was on the experimental ration. It is perhaps not surprising, after all, that demyelination resulted in these animals in the presence of an adequate supply of the antineuritic vitamin. This occurrence was shown to be possible in our earlier study already referred to (7). Moreover, it has been shown that an absence of vitamin A alone from the diet can produce demyelination of the peripheral nerves (13).

That the dogs employed in the present study were not suffering

from lack of the antineuritic vitamin is further evident from the fact that none of these animals developed the clinical manifestations of this deficiency.   As is well known, some of these neuromuscular manifestations consist of spastic paralysis, opisthotonus and convulsions, none of which signs were shown by any of the dogs in the present study.   Also, the majority of animals develop these signs of vitamin B_1 deficiency in from 60 to 90 days and succumb soon thereafter; whereas the animals in the present study lived from 200 to over 300 days.

Only after the completion of the anatomic study of the nervous system was the fact revealed that the most characteristic lesion produced by this vitamin B_2 deficiency was the degeneration of the fiber tracts of the posterior columns.   This lesion was so like that of tabes dorsalis in man that the necessity of a complete study of the reflexes and gait seemed indicated.   Also, in a recent paper, Strauss and Castle (14) had predicted the presence of anemia and achlorhydria in animals deprived of the anti-pellagra vitamin.   Unfortunately, none of these features had been adequately investigated in the present study; hence a new experiment designed essentially on the same lines as the present one has been started for this purpose, and the results will be reported later.

SUMMARY AND CONCLUSIONS

Adult dogs maintained on an artificial, balanced ration adequate in all dietary essentials as far as is known except water-soluble, heat-stable vitamin B_2 (G) developed, after a sufficient time, a slowly progressive disease characterized by loss of weight, persistent vomiting and diarrhea, and marked muscular weakness, which ended fatally in from 200 to over 300 days.

The clinical features of this condition, as pointed out in the discussion, are quite different from those characterizing the canine disease known as black tongue.

The anatomic changes in this condition consist of marked demyelination of the peripheral nerves, including the vagus; degeneration of the medullary sheaths and replacement by gliosis of the posterior columns of the spinal cord, particularly the fasciculi graciles; degeneration of the medullary sheaths of the posterior and less often of the

anterior nerve roots of the cord; occasionally slight degenerative changes in most of the other fiber tracts of the cord.

Attention is called to the fact that degenerative lesions in the central nervous system similar or identical with these have frequently been described in pellagra in man.

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EXPLANATION OF PLATES

PLATE 4

Fig. 1. Dog 6. Photomicrograph of brachial plexus. Marchi stain. × 125.

Fig. 2. Dog 6. Photomicrograph of brachial plexus showing phagocytosed fat in interstitial connective tissue. Sudan III stain. × 250.

Fig. 3. Dog 6. Photomicrograph of brachial plexus showing disappearance of many of the medullary sheaths. Spielmeyer stain. × 125.
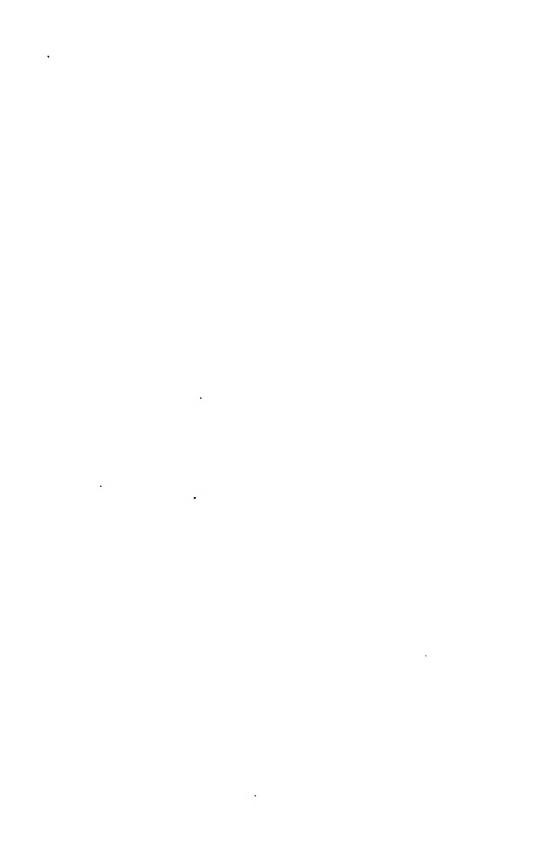
PLATE 5
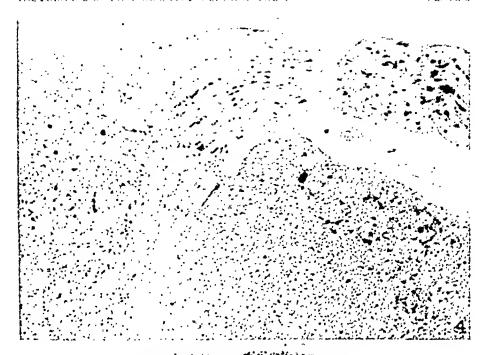
Fig. 4. Dog 5. Photomicrograph of posterior nerve root of spinal cord. Marchi stain. × 60.

Fig. 5. Dog 6. Photomicrograph of posterior columns of thoracic portion of spinal cord. The funiculi graciles are more severely injured than the funiculi cuneati. Marchi stain. × 55.
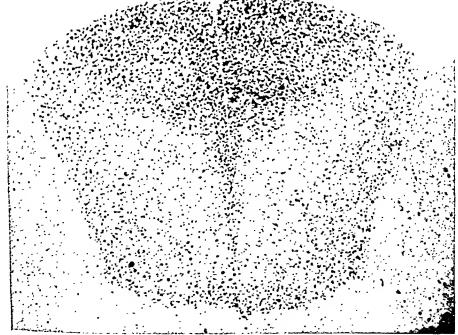
PLATE 6

FIG. 6. Dog 7. Photomicrograph of posterior columns of lumbar portion of spinal cord. Note degenerated fibers coursing into posterior horns. Marchi stain. × 23.

FIG. 7. Dog 7. Photomicrograph of longitudinal section of spinal cord. Marchi stain. × 75.
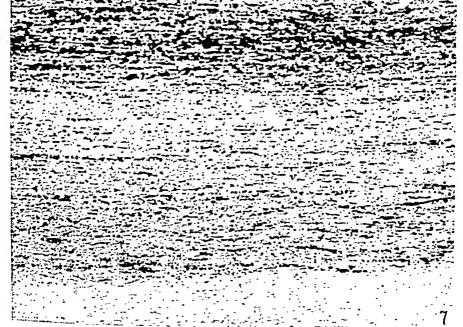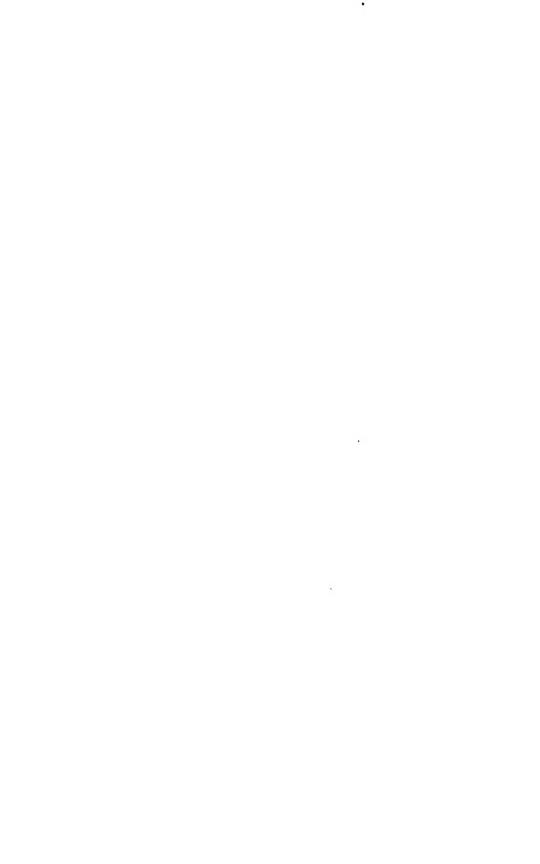
NUTRITIONAL MYOPATHY IN DUCKLINGS*

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With the Assistance of Anna Alexieff

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PLATES 7 AND 8

It has been shown in previous papers (1–4) that young chickens on a simplified diet composed of skimmed milk powder, casein, corn-starch, lard, salts, cod liver oil, yeast, and paper pulp, develop a striking disorder of the central nervous system which we have called nutritional encephalomalacia. When this same diet is given to ducklings, no changes are produced in the central nervous system, but they succumb to a remarkable and practically universal degeneration of the skeletal muscles. No other organs or tissues appear to be affected.

EXPERIMENTAL

Day old Pekin ducklings were kept in battery brooders for about 2 weeks, and then transferred to larger cages with adequate water pans. 32 experimental animals received Diet 108,[1] to which water was added to make a paste. 15 normal controls were given the Stock Diet 20.[2]

[1] Diet 108 is composed of:

	per cent
Skimmed milk powder (Merrell-Soule)	15.0
Casein (Merck's technical)	20.5
Corn-starch	20.0
Lard	21.0
Cod liver oil (Mead Johnson and Co.)	2.0
Yeast (Fleischmann's bakers', dried)	5.0
Salt mixture (McCollum 185)	6.5
Paper pulp (Eastman)	10.0

For the first 2 weeks, the behavior and appearance of the two groups were much alike. The ducks on the simplified diet showed less yellow pigmentation of bill and legs, but they were active and grew fairly well (Chart 1). Quite suddenly in the 2nd or 3rd week, symptoms of extreme weakness made their appearance. The birds were found sprawled flat, or in the earliest stages, walked awkwardly with feet turned in, and sometimes overlapping. When placed on their back, they had difficulty in righting themselves, and lay passively. As the weakness progressed, they could not raise the head from the table. The eyes were somewhat sunken. Sometimes there were coarse tremors and athetoid movements, but, in contrast to the encephalomalacic chicks, the head was not retracted and there were no spasticity, no forced movements, and no stupor. Indeed, aside from the extreme weakness, the ducklings seemed not very ill; they appeared conscious of their surroundings, free from pain, and not at all irritable. There was usually a terminal loss of weight, probably from inability to obtain food.

Most of the ducklings were sacrificed and studied soon after the onset of symptoms; some were found dead, without previous symptoms having been noted. After the symptoms were well established, recovery did not occur.

Pathology.—The subcutaneous fat and intra-abdominal fat was well preserved. Only the skeletal muscles showed pathologic changes. The most obvious feature was their pale color—often creamy

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[2] Diet 20 is composed of:

	per cent
Wheat bran	16.2
Wheat middlings	12.2
Yellow corn-meal	22.2
Ground oats	11.0
Alfalfa leaf meal	6.0
Skimmed milk powder	18.0
Cod liver oil	2.0
Yeast	2.0
Meat scraps	4.0
Bone meal	4.0
$CaCO_3$	1.6
NaCl	0.8

yellow rather than dark red. In the gross, not all the muscles were equally affected, nor was the alteration in the color always extreme. The muscle tissue was watery and translucent, the contractility impaired, but not usually wholly lost.
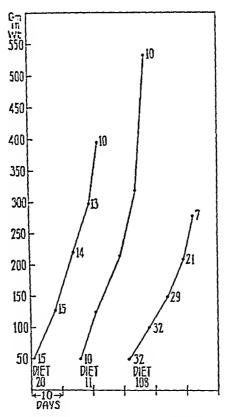
CHART 1. Composite growth curves of ducklings for the first 22 days on Diets 20, 11, and 108. The numerals at the right of each curve indicate the number of survivors.

Microscopically, there was a varying proportion of necrotic fibers, showing the usual picture of hyaline or waxy degeneration. In the most acute cases, there was little or no cellular reaction, but the intermuscular tissue was edematous. In cases which survived longer, there was an extreme cellular response in which myoblasts, histiocytes, and leucocytes of different types took part. Occasionally, basophilic

regenerating myocytes were present. The animals did not survive long enough for the necrotic fibers to become replaced by connective tissue or fat. Calcium deposits were not seen. The lesions are illustrated in Figs. 1 and 2. The brain was examined in all the cases, and the spinal cord and sciatic nerves in many. No significant lesions were found. A study of the motor nerve endings by Rogers' method showed, as in the nutritional muscular dystrophy of rabbits and guinea pigs (5), that these structures were not affected (Fig. 3). The only incidental lesions noted in a few animals were erosions of the gizzard mucosa, and in one case a perforating ulcer with peritonitis. The myocardium and smooth muscles were free of change, and nothing abnormal was seen in the other viscera. No detailed histological study has been made of the bones but there were no gross deformities. The epiphyseal cartilages were not widened, and the bones cut with great resistance.

The controls grew well and when killed at corresponding intervals showed normal muscles and viscera.

Cultures (from heart, liver, spleen, and in some animals from the muscle tissue) were made on infusion agar slants, and incubated for 7 days at 37°C. The results are shown in Table I and offer no evidence of an infectious etiology. No bacteria or other microorganisms were demonstrable in the lesions.

Of the 32 ducklings on Diet 108, 3 were found dead. 9 others presented typical myasthenic symptoms, but at autopsy, the muscle changes escaped notice, and the muscles were not sectioned for histological study. The observations upon the remaining 20 ducks are summarized in Table I, from which it can be noted that 17 showed pronounced and obvious symptoms and lesions. There were 3 negative cases. No. D-1895, which was killed on the 30th day because of weakness and prostration, proved to have perforating ulcers of the gizzard, but there were no muscle lesions, and the muscle creatine was within normal limits. No. D-1924 died on the 11th day of a generalized infection, probably due to an organism of the paratyphoid group. No. D-1929 was killed on the 19th day because it walked awkwardly and seemed weak on its legs. Although the muscle creatine was distinctly low, there were no histological changes in the muscle.

In a previous study (6) it has been shown that there is a decrease in the creatine content of the muscles in nutritional muscle dystrophy of rabbits. Similarly in this disease of ducks, the creatine content of the degenerated muscle was reduced.

The individual values for the muscle creatine, as determined by the Rose, Helmer, and Chanutin modification of the Folin method (7) are listed in Table I. In 17 normal ducklings of the same age, the muscle creatine was found to have a mean value of 415 mg. creatine per 100 gm. fresh tissue, with a standard deviation

TABLE I

Effect of Diet 108 upon Ducks

Duck No.	Period on diet	Myasthenic symptoms	Microscopic lesions		Analysis of leg muscle		Remarks
			Central nervous system	Muscles	Moisture	Creatine per 100 gm. fresh tissue	
	days				*per cent*	*mg.*	
1888	K 30	+	—	+++	80 6	310	
1889	K 22	+++	—	+++	77 3	217	
1891	K 24	+++	—	++			
1893	K 19	+++	—	+			
1895	K 30	+++	—	—	77 8	418	Perforating ulcer of gizzard
1898	K 20	+++	—	+++	82 0	301	
1899	K 18	+++	—	+++			
1900	K 20	+++	—	++++	76 9	288	
1902	D 27		—	+++	78.7	236	
1919	K 18	++	—	++++	81 8	243	
1920	K 21	+++	—	++++	82 8	103	
1921	K 14	++	—	++++	—	128	Cultures sterile
1922	K 19	+++	—	++	79.4	187	Liver culture—large Gram-positive diplococcus; spleen sterile
1923	K 33	++	—	++	80.7	302	
1924	D 11		—	—	—	186	*B. paratyphosus* from spleen, liver, and heart blood
1925	D 14	+++	—	++	—	547	
1926	K 16	++	—	++++	82.2	88	
1927	K 19	+++	—	+++	82.9	179	Cultures from leg muscle—sterile; liver Gram-positive bacilli. Erosions of gizzard
1928	K 15	+++	—	++++	82.6	106	Cultures sterile
1929	K 19	+	—	—	77.6	288	Cultures sterile

of ±56; the probable error of the mean was ±9.2, and that of the standard deviation, ±6.9. Although the creatine content of normal muscle varies greatly, the values given in the table for degenerated muscle are seen to be significantly low, ranging from 88 to 310 mg.

The only exception is No. D-1925, in which fairly marked lesions were present with a creatine of 547 mg. However, different blocks of muscles in this animal showed great variation in the intensity of the histological changes, one being normal, and the sample taken for creatine determination may not have been representative.

Moisture determinations were also made. The mean value obtained in 17 normal ducklings was 78.3 per cent with a standard deviation of ±1.8; the probable error of the mean was ±0.3 and that of the standard deviation was 0.2. It is apparent from the table that in many instances, the moisture content of the degenerated muscle was increased, a fact which is in accord with the observed edema.

Studies of the oxygen consumption and irritability were made by Dr. Joseph Victor and will be reported in a separate communication.

The resemblance of these muscle lesions to those which are produced in rabbits or guinea pigs by nutritional means (8) led to an experiment in which 10 ducklings were given Diet 11.[3] Although some of the animals showed a certain awkwardness in gait, in a few instances, so severe as to incapacitate them, this seemed to be associated with deformity of the bones instead of muscle weakness or disease of the central nervous system. Most of the birds showed excellent growth, and only in the 4 so crippled that they had difficulty in obtaining nourishment did death result.

The muscular system, both grossly and microscopically, was not affected. The creatine content of the muscle was within normal limits. The brain, cord, and peripheral nerves were also unaffected. The bones were not subjected to careful study. In one instance, however, rachitis-like changes—widening and irregularity of the cartilage, slight excess of osteoid, and fibrosis of the marrow, were present in the upper extremity of the tibiotarsus.

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[3] Diet 11 is composed of:

	per cent
Rolled oats	35.5
Wheat bran	12.0
Casein (Merck's technical)	7.5
Lard	8.0
Cod liver oil (Mead Johnson and Co)	1.0
NaCl	1.0
CaCO₃	1.5
Skimmed milk powder	27.5

DISCUSSION

The most interesting aspect of these observations is that the same diet produces in two different species of birds widely different pathological effects. In chickens, the injurious action is limited to the brain —in ducks, to the voluntary muscles. In both cases, the lesions are associated with characteristic clinical symptoms.

It would be futile at this time to explain either condition in terms of known dietary factors, or to speculate as to why such dramatically diverse changes should occur on the same diet in the two species.

In spite of the close resemblance between the pathological lesions in the muscles of ducks on Diet 108 and those produced by Madsen, McKay, and Maynard (9) in goats, rabbits, and guinea pigs on synthetic diets, it would be unwise to assume that the same nutritional fault is responsible. The fact that Diet 11, which leads to severe muscle degeneration in rabbits and guinea pigs, is without effect upon the muscles of ducks makes the identity of the avian and mammalian disease very doubtful.

CONCLUSIONS

Ducklings fed on a diet of skimmed milk powder, casein, corn-starch, lard, cod liver oil, yeast, salts and paper pulp rapidly develop a disease characterized by extreme and progressive myasthenia, ending in death within a few days.

Pathological changes are found in the skeletal muscles. These show widespread hyaline necrosis of fibers, with edema and cellular reaction. The brain and other parts of the central nervous system are not affected, and no significant alterations are found in other viscera or tissues. The creatine content of the muscles is reduced in proportion to the muscle injury. Controls on a natural food diet remain free from the disease.

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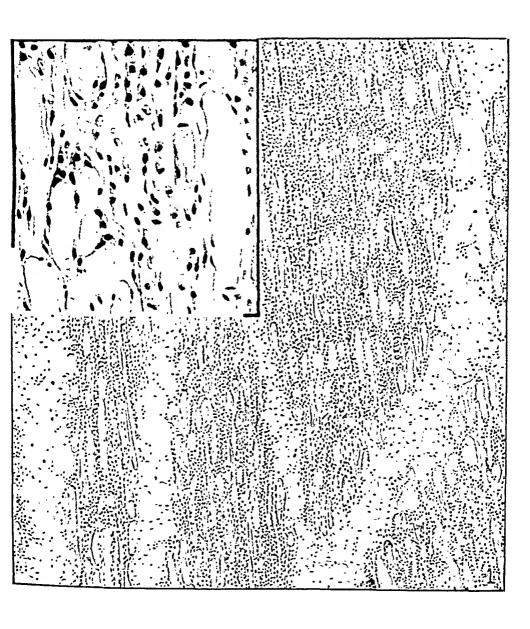
EXPLANATION OF PLATES

PLATE 7

FIG. 1. Duck 1898. Killed after 20 days on Diet 108; complete prostration. Gluteus muscle. Extreme waxy necrosis. The degenerated fibers are surrounded by myoblasts and histiocytes; the stroma infiltrated with great numbers of mononuclear and polymorphonuclear leucocytes.

PLATE 8
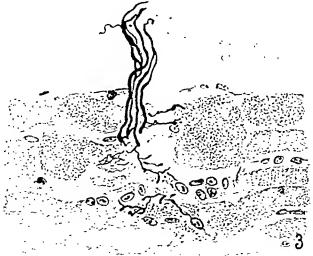
FIG. 2. Duck 1921. Killed after 14 days on Diet 108. Extensor muscles of leg. Extreme necrosis and fragmentation of fibers with interstitial edema. Little cellular reaction. Insert shows higher magnification.

FIG. 3. Duck 1926. Killed after 16 days on Diet 108, when unable to stand erect. Gluteus muscle—Rogers stain. In spite of extreme necrosis of fibers, the neurites and motor end plates are normal in appearance.

PLATE 8

AN INVESTIGATION INTO THE SIGNIFICANCE OF HORMONAL FACTORS IN EXPERIMENTAL POLIOMYELITIS*

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Poliomyelitis continues to be a formidable problem in clinical medicine and public health. Although no one would today seriously question the infectious character of the disease, our knowledge of its pathogenesis is still imperfect. Thus, insusceptibility to the virus, exemplified by the extent of racial and individual resistance, is so widespread that an attack of poliomyelitis after known exposure is the exception rather than the rule. Again, while the effect of age, season and climate on the outbreak of epidemics of poliomyelitis is well established, the manner in which these factors affect the mechanism of resistance to the disease remains obscure. The irregular spread of the infection has been interpreted as indicating a wide dissemination of the virus among civilized populations, with the further inference that a majority of adult individuals can carry it without ill effects. Precisely where one should look for the source of this insusceptibility, however, is a question on which opinions are sharply divided. Most authorities incline to the belief that the protection is due to an almost universal process of specific immunization against the infectious agent, brought about either by abortive attacks of the disease (Wickman (1); Paul, Salinger and Trask (2)) or by latent epidemization (Wernstedt (3); Aycock (4)). The arguments which support these hypotheses have been gathered largely from epidemiological observations. While the various data taken at their face value, particularly when viewed in the light of analogy with other infectious diseases, are suggestive, the evidence can by no means be considered as conclusive. On

analysis many inconsistencies become apparent, which we have discussed in greater detail in a recent critical review (5).

The inadequacy of the present hypotheses has prompted us to serious consideration of the merits of a simpler conception involving fewer unproved premises; namely, that the predeliction of the disease for the prepubertal age groups may indicate the absence in the child of certain physiological factors which, in their totality, are responsible for the difference between the immature and mature organism. In experimental poliomyelitis, the importance of the age factor is suggested by statements in the earlier and recent literature that younger monkeys are more susceptible to infection than are older ones (Landsteiner (6); Levaditi (7); Leiner and von Wiesner (8); Pette (9); Flexner (10)). As a matter of fact, most of the monkeys commonly available for experimental purposes are infantile or immature animals, ranging in weight from 5 to 8 pounds, and exhibit no signs of somatic or sexual maturity. The males still carry the testes in the abdominal cavity or within the inguinal ring, with a low degree of spermatogenic activity. The females rarely show reddening of the sexual skin or any other evidence of sexual maturity, the condition of the ovaries and uterus being distinctly prepubertal.

It seemed important, therefore, first to investigate more systematically whether the experimental infection in adult or subadult monkeys would proceed in a course different from that observed in immature animals. Secondly, we decided to determine whether it was possible to obtain any evidence for enhanced resistance to poliomyelitic infection in monkeys in which attempts had been made to induce in a sense a precocious physiological maturity by treatment with certain endocrine principles. This artificial change of endocrine balance could be accomplished experimentally in a variety of ways. Since the pioneering work of Philip Smith (11), Evans and coworkers (12), Smith and Engle (13), and Zondek and Aschheim (14), the anterior lobe of the pituitary gland has come to be regarded not only as the "motor" of the genital system but also as an organ which has important functions for the growth of the body and the maintenance of intermediary metabolism. Another possibility of substituting a single phase of sexual maturity only was opened up by the application of principles evolved from the work of Allen and Doisy (15) on the female

sex hormone.   Finally, in order to provide for a fair basis of comparison of such non-specifically enhanced resistance with the protection acquired by specific immunity, we have added some experiments on active immunization of monkeys by poliomyelitis virus.   The progress of this investigation is presented in the following pages in three separate sections.

Experimental Poliomyelitis in Adult or Subadult M. rhesus and Virucidal Tests with the Normal Serum of Adult or Subadult Monkeys and Apes

Experimental poliomyelitis was produced in a series of six adult or subadult *rhesus* monkeys and one new-born monkey, employing the usual technique of intracerebral infection.   The dose of virus ranged from 0.1 to 1 cc. of a 10 per cent virus emulsion.   Virucidal tests with the normal serum of the above animals and of two additional subadult monkeys were carried out simultaneously in the routine manner, the proportions of virus and serum in the different mixtures being adjusted to a greater or lesser severity.   Infection experiments and virucidal tests were accompanied by adequate controls.   The results of this work, together with all technical details, appear in Table I.

A study of Table I shows that poliomyelitic infection in adult *rhesus* monkeys runs frequently a course more protracted than that observed in immature animals, particularly when small doses of inoculum are used.   Thus in three adult animals the incubation period was doubled or nearly doubled as compared with the controls, while in two other adult monkeys the severity of the infection was the same as with the immature animals.   The above mentioned differences in the type of infection are comparatively slight but nevertheless noteworthy since the strain of passage virus used in this work for almost 4 years has now acquired a uniform degree of infectivity with a relatively stable incubation period resembling that of rabies fixed virus. It is possible that greater differences might have been realized by a further reduction of the infecting dose.   Of particular interest is the result obtained with Monkey 6 and its newly delivered young.   We were fortunate in obtaining this animal 2 days after it had given birth to a young one.   Mother and new-born proved completely insusceptible to intracerebral infection with a potent dose of virus.   The striking analogy, in this case, between animal experiment and empirical observations in man is obvious.   The results of the virucidal

TABLE I

Experimental Poliomyelitis in Subadult or Adult rhesus Monkeys

Monkey No.	Weight	Sex	Infection	Virucidal test with normal serum			
				Monkey No.	Amount of virus	Amount of serum	Result
	lbs.				cc.	cc.	
1	15	F	*Complete paralysis, 14 days	H40	0.2	0.8	No paralysis
Control H22	5	F	* " 8 "				
2	18	F	* " 13 "	J70	0.2	0.8	Complete paralysis, 11 days
Control J40	5	F	* " 5 "				
3	23	M	* " 9 "	J71	0.2	0.8	No paralysis
Control J40	5	F	* " 5 "				
4	21	M	* " 14 "	J48	0.2	0.8	Complete paralysis, 10 days
Control J40	5	F	* " 5 "				
5	16	F	† " 7 "	G12	0.6	0.6	Complete paralysis, 6 days
Control G11	6	F	† " 6 "				
6 (mother)	14	F	‡ No paralysis	I94	0.1	0.9	Complete paralysis, 13 days
(young)	3/4	M	‡ " "	I95	0.1	0.6	" " 13 "
Control I96	5	F	‡ Complete paralysis, 5 days				
7	12	F	Not done	F47	0.6	0.6	Complete paralysis, 13 days
8	12	F	" "	F48	0.6	0.6	" " 10 "

* Monkeys marked thus received 0.2 cc. 10 per cent virus + 0.8 cc. saline intracerebrally.

† Monkeys marked thus received 0.6 cc. 10 per cent virus intracerebrally.

‡ Monkeys marked thus received 0.1 cc. 10 per cent virus + 0.9 cc. saline intracerebrally.

Technique of Virucidal Test.—Serum and virus suspension were combined in the stated amounts. The mixture was kept 1½ hours at 37°C. and overnight in the ice box. 1 cc. was injected intracerebrally.

Controls for Virucidal Tests:

H22 (for H40). Complete paralysis, 8 days.   J69 (for J70, J71). Complete paralysis, 9 days.

J40 (for J48). Complete paralysis, 5 days.   G11 (for G12). Complete paralysis, 6 days.

I96 (for I94, I95). Complete paralysis, 5 days.   F40 (for F47, F48). Complete paralysis, 6 days.

tests of the corresponding normal sera in this series are in good agreement with the outcome of the infection experiments. Thus the serum of two adult animals completely neutralized the virus *in vitro* while the greatly prolonged incubation periods with the serum of another subadult monkey and with the serum of the *rhesus* mother and her young suggest some degree of neutralizing activity. These observations are therefore confirmatory of earlier experiments, in which

TABLE II

Virucidal Tests with the Normal Sera of Subadult Chimpanzees

Chimpanzee	Weight	Sex	Approximate age	Virucidal test			
				Monkey No.	Amount of serum	Amount of 10 per cent virus	Result
	lbs.		*yrs.*		*cc.*	*cc.*	
C	70	M	7	G73	0.6	0.6	Complete paralysis, 26 days
				H4	0.8	0.2	No paralysis
J	57	M	6	G74	0.6	0.6	Slight paresis, one leg, 12 days
M	62	F	5–6	G86	0.6	0.6	Complete paralysis, 8 days

Technique of virucidal test as previously indicated.
Controls:
G61 (for G73, G74). Complete paralysis, 6 days.
H2, H1 (for H4). Complete paralysis, 7 days; almost complete paralysis, 5 days.
G79, G78 (for G86). Complete paralysis, 7 days.

we described the occasional neutralization of the virus *in vitro* with the serum of mature *rhesus* monkeys (16).

Through the kindness of Dr. Dochez and his associates, Dr. Kneeland and Miss Mills, an opportunity was afforded to examine the sera of three subadult chimpanzees for poliocidal properties.[1] The results which are recorded in Table II, indicate clearly that the normal serum

[1] Pettit and his associates (17) reported that they found the serum of a normal adult female chimpanzee devoid of virucidal power. The weight of this animal was given as about 30 kilos, the age was not stated definitely in years. It would seem that this particular ape was of about the same age as our youngest animal.

of the oldest ape completely inactivated small doses of virus, while the serum of the animal next in age possessed enough virucidal power to produce a partial neutralization effect. In contrast to these two older chimpanzees, the serum of the youngest animal was devoid of virucidal power in the proportions tested. These experiments offer a good analogy to similar data obtained with the sera of human populations presumably free from epidemic poliomyelitis (18) and support the concept of the physiological origin of the virucidal principle in normal human serum.

Influence of Preparation with Various Endocrine Principles on the Resistance of the rhesus Monkey to Poliomyelitic Infection and on the Virucidal Power of the Serum

(a) Effect of Implantation of the Anterior Pituitary Lobe from Adult Rats

The most active principle capable of accelerating somatic development is the internal secretion of the anterior lobe of the pituitary. While many extracts have been prepared by chemical manipulation, none have duplicated the conditions obtained in rats and mice by the daily implant of fresh glands from adult animals. Anterior pituitary implants from rats, therefore, were administered to a small group of five monkeys before infection, according to the method devised by Smith (11).

The animals received intramuscularly in the thigh daily implants of from four to seven pooled anterior lobes of adult female or male rats during a period of from 8 to 15 days. The response of the animals was in general similar to that observed after treatment with anterior pituitary extract (Engle (19)). After completion of this course of preparation, the animals were inoculated intracerebrally with 1 cc. of a 10 per cent virus suspension, together with an adequate number of controls. The results of these experiments are brought together in Table III.

As can be seen from this table, there was a pronounced difference in the course of infection in two of the prepared animals, as indicated by the lightness of the symptoms and the prolonged incubation period when compared with the corresponding controls. Three other prepared animals, however, succumbed to the infection as rapidly as their controls and the disease had the same severity. The experimental evidence therefore, while perhaps suggestive, was far from conclusive.

(b) *Effect of Female Sex Hormone*

Recent investigations on the effect of various preparations of female sex hormone on the reproductive organs of rodents and monkeys suggested a trial of these substances in our work. The two products which have been best studied, experimentally and clinically, are amniotin (Squibb) and theelin (Parke, Davis).[2] Amniotin prepared from the amniotic fluid of cattle is known to induce estrus in castrated rats and mice; in the immature monkey it produces after 6 or 7 days a redden-

TABLE III

Effect of Preparation with Anterior Pituitary Rat Implants on the Course of Experimental Poliomyelitis

Monkey No.	Duration of preparation	Dosage daily	Infection
			1 cc. 10 per cent virus intracerebrally
	days		
C27	15	5 lobes	Slight paresis one leg, 13 days
C81	15	4 "	Complete paralysis, 6 days
D7	15	7 "	Partial paralysis one arm, 10 days
D42	15	5 "	Complete paralysis, 7 days
D66	8	5 "	" " 8 "

Controls:
C49 (for C27).   Complete paralysis, 5 days.
C96 (for C81).   Complete paralysis, 7 days.
D12 (for D7).   Complete paralysis, 6 days.
D57 (for D42 and D66).   Complete paralysis, 5 days.

ing of the sexual skin, swelling and edema of the external genitals and a considerable hypertrophy of the uterine endometrium with a variable premenstrual glandular development (Allen, Morrell, Engle, *et al.* (20)).   Theelin, prepared in crystalline form from the urine of pregnant women, has the standard estrogenic properties in spayed female rats and on the whole, causes changes in the monkey similar to those described for amniotin.   Neither of the two hormones affects the size of the ovary, save that after prolonged treatment with massive doses the organ may be found to be actually smaller.

[2] We are indebted to Dr. O. P. Kamm of Parke, Davis and Co., and to Dr. J. F. Anderson of E. R. Squibb and Sons for placing at our disposal generous amounts of these preparations.

Our experiments with these two hormones comprise a series of fourteen monkeys, of which eleven were prepared by subcutaneous injections with theelin and the remaining three with amniotin (see Table IV). All animals were then injected intracerebrally with a potent dose of a 10 per cent virus suspension. While a correct interpretation of our observations is difficult because of the limited number of experimental animals, the trend of the results would indicate that, with a few exceptions, the prophylactic administration of either theelin or amniotin had little or no demonstrable effect on the severity of the infection as compared with the controls. This opinion is further strengthened by the failure of theelin-treated animals to develop any virucidal principles in the serum. Whether the lighter infection observed in three theelin-prepared animals was due to chance or whether some other as yet uncontrollable factor was responsible for this effect must remain undetermined. At any rate, the results were not deemed sufficiently encouraging to stimulate further investigation of this type of hormone.

(c) Effect of Extracts Prepared from Sheep Anterior Pituitary Glands

Pyridine extracts prepared from dried powder of anterior pituitary sheep glands have proved a potent source of hormones, active in enhancement of growth and stimulation of the reproductive system (Hisaw et al. (21) and Engle (22)).

A total of seven immature monkeys of either sex were treated by daily injection with pyridine extracts prepared from powdered anterior pituitary sheep glands for periods of time varying from 9 to 16 days. After completion of this preparation the animals were inoculated intracerebrally with 1 cc. of 10 per cent virus suspension and, at the same time, their serum was tested for virucidal power in the usual manner. This experimental series together with the accompanying controls is listed in Table V.

It will be seen from Table V that only one animal resisted intracerebral infection after preparation while the remaining animals succumbed to the infection like the corresponding controls. However, the results of the virucidal tests with the sera of the prepared animals were more clear-cut. Thus, four sera completely inactivated the virus *in vitro* and the serum of a fifth animal should be listed as

TABLE IV

Effect of Preparation with Female Sex Hormone (Theelin, Amniotin) on Course of Experimental Poliomyelitis

Monkey No.	Preparation	Duration of preparation	Dosage daily	Infection	Virucidal test with serum after preparation	
					Monkey No.	0.2 cc. 10 per cent virus + 0.8 cc. serum
		days	cc.			
C28	Theelin	9	2	* Complete paralysis, 9 days		
C29	"	9	2	* Paresis one leg, 9 days		
D4	"	9	2	* " " " 9 "		
D6	"	9	2	* " " " 7 "		
D35	"	15	2	* Complete paralysis, 7 days		
D36	"	15	2	* " " 9 "	D48	Partial paralysis, 8 days
D37	"	15	2	* " " 8 "		
D38	"	15	2	* " " 8 "		
D55	"	8	2	* " " 6 "		
H72	"	10	1	† " " 7 "	I10	Compl. paralysis, 8 days
H80	"	10	1	† " " 6 "	I11	Paresis one leg, 6 days
C71	Amniotin	20	4	* " " 5 "		
C76	"	20	4	* " " 7 "		
C82	"	20	4	* " " 5 "		

* Monkeys thus marked received 1 cc. of 10 per cent virus suspension intracerebrally.

† Monkeys thus marked received 0.2 cc. of 10 per cent virus suspension + 0.8 cc. saline intracerebrally.

Technique of virucidal test as previously indicated.

Controls:

C49 (for C28).   Complete paralysis, 5 days.

C54 (for C29).   Complete paralysis, 7 days.

D11 (for D4, D6).   Complete paralysis, 5 days.

D39, D40 (for D35, D36, D37, D38).   Complete paralysis, 8 days; complete paralysis, 6 days.

D56 (for D55).   Complete paralysis, 8 days.

I8 (for H72, H80, I10, I11).   Complete paralysis, 7 days.

C96 (for C71, C76, C82).   Complete paralysis, 7 days.

D46 (for D48).   Complete paralysis, 7 days.

partially neutralizing, leaving only two sera which were devoid of virucidal power in the amounts tested.

(d) *Effect of Preparation with Prolan on Resistance to Experimental Infection and on the Virucidal Property of the Serum*

By far the largest number of animals will be found in this section. We have emphasized this particular phase of our investigation because of the unique advantages attending the use of anterior pituitary-like principles of human origin obtained in rich concentrations by proper extraction from the urine of pregnant women. While pregnancy urine apparently contains a number of biologically and chemically different hormones, the presence of a gonad-stimulating hormone is recognized as one of the chief components. This particular substance, following the nomenclature of Zondek and Aschheim, is called prolan.

Prolan was prepared in our laboratories from large amounts of urine collected from women in the late and earlier stages of pregnancy, by two methods. The first one consisted in precipitation of the active principle by alcohol, the other by adsorbing it to benzoic acid. Either method yielded preparations high in ovary-stimulating activity when tested in the mouse or rabbit. These products as a rule were prepared so that the final solution represented a concentration of 1:40 compared with the volume of the original material.

A total of twenty-three immature monkeys of either male or female sex were prepared by daily subcutaneous injections of 2 to 8 cc. of prolan for periods of time ranging from 9 days to 1 month. During this preparation the animals showed few outward physical changes. Data relating to the response of monkeys to prolan treatment are published elsewhere (Engle (22)). Suffice it to state here that prolan is not a strong ovarian activator in the monkey, and fails to induce the estral response characteristic of glandular anterior pituitary principles. However, it does cause activation of the interstitial cells in the testes. After completion of this preparation, the animals were infected intracerebrally with 1 cc. of a 10 per cent virus suspension; simultaneously virucidal tests were carried out to determine the capacity of the sera to inactivate the virus *in vitro*. The protocols of this experimental series, including proper controls, appear in Table VI.

A study of Table VI shows that protection against intracerebral infection can be obtained only in exceptional cases in monkeys prepared with prolan. Thus, only two animals proved refractory to the infection and two additional monkeys went through a very slight, abortive type of the disease after greatly prolonged incubation periods. In contrast to this, the results obtained from virucidal tests were far

superior. Of a total of twenty-nine different samples of serum from prepared animals, ten inactivated the virus completely and five other samples should be considered as possessing partial neutralizing power, judging from the lightness of the resulting infection or the marked pro-

TABLE V

Effect of Preparation with Extracts Prepared from Sheep Anterior Pituitary Gland on Experimental Infection and the Virucidal Property of the Serum

Monkey No.	Dura- tion of prepa- ration	Dos- age* daily	Infection	Virucidal test with serum after preparation	
			1 cc. 10 per cent virus suspension i.c.	Mon- key No.	Result 0.6 cc. serum + 0.6 cc. 10 per cent virus
	days	*cc.*			
D71	9	2	No paralysis	D85	No paralysis
E1	16	2	Complete paralysis, 11 days	E24	" "
E8	10	2	" " 7 "	E18	" "
E91	14	2	Died before infection	E39	" "
E10	10	2	Complete paralysis, 9 days	E19	Complete paralysis, 8 days
E93	14	2	" " 10 "	E73	" " 11 "
E57	10	3	Partial " 8 "	D43	" " 13 "

* The extracts were prepared so that 2 cc. contained the equivalent of 0.6 gm. of dried pituitary.

Technique of virucidal test as previously indicated.

Controls:

D69 (for D71). Complete paralysis, 10 days.

E15 (for E1, E8, E10). Complete paralysis, 7 days.

F17 (for E93, E73). Complete paralysis, 5 days.

E69 (for E57). Complete paralysis, 7 days.

E23 (for E24, E18, E19). Complete paralysis, 7 days.

E34, E35 (for E39). Complete paralysis, 6 days.

E82 (for D43). Complete paralysis, 6 days.

D79 (for D85). Complete paralysis, 10 days.

longation of the incubation periods. In other words, about one-half of the sera showed some degree of poliocidal power after preparation with prolan. One may feel reasonably assured that this property did not exist in that intensity before injection of the hormone since there is a general concensus of opinion in the literature that the normal serum of immature *rhesus* monkeys fails uniformly to inactivate the virus

Effect of Preparation with Prolan on Resistan

Prepared Monkey No.	Duration of preparation	Dosage of prolan daily	Intracerebral infection		
			Result in prepared monkey	Control Monkey No.	Res
	days	*cc.*			
P1	9	8	Complete paralysis, 7 days	D11	Comp
P2	9	8	Partial " 6 "	D11	"
P3	20	8	No paralysis	C96	"
P4	20	8	Partial paralysis, 16 days	F40	"
P5	20	8	Complete " 10 "	F40	"
P6	30	2	Died 6th day without symptoms	G11	Compl
P7	30	2	Complete paralysis, 6 days	G11	"
PS	30	2	Complete paralysis, 6 days	G11	Compl
P9	30	2	Complete paralysis, 7 days	G11	Compl
P10	30	2	Died 2nd day without symptoms	G11	Compl
P11	30	4	Complete paralysis, 7 days	G11	Compl
P12	30	4	Died before infection		
P13	30	4	Complete paralysis, 6 days	G61	Compl
P14	30	4	Died before infection		
P15	30	4	Died before infection		
P16	30	4	Died before infection		
P17	30	4	No paralysis	H2	Comple
P18	30	4	Died before infection		
P19	30	4	Complete paralysis, 10 days	H2	Comple
P20	30	4	Died before infection		
P21	30	4	" " "		
P22	15	5	Complete paralysis, 8 days	I21	Comple
P23	15	5	Partial " 14 "	I21	"

All prepared animals received 1 cc. of 10 per cent virus suspension.

All control monkeys received 0.6 cc. of 10 per cent virus suspension + 0.6 cc. saline or ne saline.

The serum of the prepared monkeys was tested in amounts of 0.6 cc. against 0.6 cc. of

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al Infection and on the Virucidal Property of the Serum

	Test Monkey No.	Result in test monkey			Control Monkey No.	Result in control monkey		
		Virucidal test with serum after preparation						
days		Not tested						
"		" "						
"		" "						
"	F4	Paralysis one arm, 16 days			F40	Complete paralysis,	6 days	
"	F38	1st bleeding. No paralysis			F31	"	"	7 "
	F44	2nd " Complete paralysis, 9 days			F40	"	"	6 "
days	G16	No paralysis			G11	"	"	6 "
"	G2	1st bleeding. Partial paralysis, 10 days			F97	Partial	"	10 "
	G20	2nd " No paralysis			G19	Complete	"	5 "
days	G5	1st " No "			F97	Partial	"	10 "
	G95	2nd " Complete paralysis, 7 days			G19	Complete	"	5 "
days	G6	1st " No paralysis			F97	Partial	"	10 "
	F90	2nd " Complete paralysis, 8 days			G19	Complete	"	5 "
days	G7	1st " Partial " 11 "			F97	Partial	"	10 "
	G96	2nd " No paralysis			G19	Complete	"	5 "
days	G15	Complete paralysis, 7 days			G11	"	"	6 "
	G62	1st bleeding. Complete paralysis, 8 days			G61	"	"	6 "
	H20	2nd " " " 7 "			H21	"	"	7 "
days	G63	Partial paralysis, 11 days			G61	"	"	6 "
	G64	1st bleeding. Partial paralysis, 11 days			G61	"	"	6 "
	G97	2nd " No paralysis			G79	"	"	7 "
	G65	1st " " "			G61	"	"	6 "
	G85	2nd " Complete paralysis, 9 days			G61	"	"	6 "
	G84	Complete paralysis, 14 days			G79	"	"	7 "
days	G83	1st bleeding. Complete paralysis, 9 days			G79	"	"	7 "
	H15	2nd " No paralysis			H22	"	"	8 "
	G82	Slight paresis arms, 9 days			G79	"	"	7 "
' days	H14	Complete paralysis, 14 days			H2	"	"	7 "
	H23	" " 7 "			H21	"	"	7 "
	H25	" " 7 "			H21	"	"	7 "
5 days	I15	No paralysis			I21	"	"	6 "
5 "	I16	Complete paralysis, 14 days			I21	"	"	6 "

.ey serum except Nos. H2, H21 and I21, which received 0.2 cc. of 10 per cent virus suspension + 0.8 cc.

t virus suspension. Technique of virucidal test as previously indicated.

when tested in the usual proportions. However, we have again satisfied ourselves in this investigation by occasional tests that the serum of experimental animals contained no demonstrable virucidal substances before hormone treatment was begun. In several instances, where the animals were bled repeatedly during the period of preparation, *i.e.* at early and late intervals, conflicting results were obtained in the virucidal tests. We cannot at present account for these irregularities inasmuch as sometimes the earlier bleeding proved the better and the later the poorer, while in other cases the reverse was true. It follows from these observations that the virucidal power of the serum developed as the result of hormonal stimulation is very irregular and fleeting, depending upon variations in responsiveness which so far are beyond experimental control.

In order to exclude the possibility that the virucidal substances in the serum of prepared monkeys owe their origin to some entirely non-specific effect following the parenteral introduction of large amounts of foreign protein, six additional monkeys were prepared in a similar manner with liver extract and with spleen extract. None of these animals were found protected against intracerebral infection and none of the sera after preparation showed any evidence of virucidal activity.

Effect of Active Immunization with Dead and Live Virus on the Resistance of the Monkey to Experimental Infection and on the Virucidal Property of the Serum

It has been shown repeatedly that active immunization with the specific agent of the disease induces in monkeys a state of immunity which can be demonstrated by resistance of the immunized animals to intracerebral infection and by the appearance of virucidal substances in the serum. The percentage of protected animals has varied considerably in the hands of different investigators according to the methods employed (23). In order to compare non-specific enhancement of resistance obtained by hormone preparation with the effect of specific immunization, we immunized a larger number of monkeys with dead or live virus and examined the animals after immunization for acquired resistance by infection and virucidal tests.

The first series comprised eight monkeys which received large doses of formalin-inactivated virus by the subcutaneous route. A total of eight injections, each

TABLE VII

Effect of Active Immunization with Live Virus and of Prolan in Combination with Virus on Experimental Poliomyelitis in Monkeys

Monkey No.	Virus, total dosage	Prolan, total dosage	Duration of immunization	Infection 0.2 cc. 10 per cent virus i.c.	Control Monkey No.	Control 0.2 cc. 10 per cent virus i.c.
			days			
H74	10 cc. 20% suspension s.c.	24 cc. s.c.	7	No paralysis	121	Complete paralysis, 6 days
H75	10 " 20% "	24 " "	7	" "	121	" , 6 "
I88	10 " 20% "	24 " "	7	Partial paralysis, 9 days	J40	" 5 "
I89	10 " 20% "	24 " "	7	Complete " 9 "	J40	" 5 "
I90	10 " 20% "	24 " "	7	" " 10 "	J40	" 5 "
I91	10 " 20% "	24 " "	7	No paralysis	J40	" 5 "
I93	10 " 20% "	24 " "	7	Partial paralysis, 10 days	J40	" 5 "
H78	10 " 20% "		7	Complete paralysis, 7 days	121	" 6 "
H79	10 " 20% "		7	" " 11 "	121	" 6 "
I83	10 " 20% "		7	" " 11 "	J40	" 5 "
I84	10 " 20% "		7	" " 11 "	J40	" 5 "
I85	10 " 20% "		7	No paralysis	J40	" 5 "
I86	10 " 20% "		7	" "	J40	" 5 "
I87	10 " 20% "		7	" "	J40	" 5 "

consisting of 5 cc. formalinized 20 per cent virus suspension, were administered over a period of 4 weeks. After a rest period of 2 weeks the animals were infected intracerebrally and their serum was tested for virucidal power *in vitro*.

None of the treated animals was protected against the infection and none of the sera showed any evidence of virucidal property.

In a second series, a total of sixteen monkeys received daily injections of 2 cc. 20 per cent live virus suspension by the subcutaneous route during a period of 1 week. Eight of these animals received in addition daily injections of prolan. This latter modification was introduced on the assumption that a more marked effect may possibly be obtained by a combination of virus and hormone. Two of the sixteen monkeys developed poliomyelitis shortly after completion of immunization. The remaining fourteen were infected after a rest period of 2 weeks by intracerebral injection of 0.2 cc. of 10 per cent virus suspension. The results of this work are presented in Table VII.

It appears from Table VII that of a total of fourteen immunized animals, six resisted the intracerebral infection and three additional animals showed only partial paralysis, while five succumbed to the infection with fully developed symptoms. Of the completely paralyzed animals, three had been immunized with virus alone and two with virus and prolan. The six fully protected animals were evenly divided between the two types of immunization. This experiment therefore permits of the conclusion that there existed no appreciable difference between the effect of virus alone and virus in conjunction with prolan, as immunizing agents. It also demonstrates that active immunization with live virus, under the conditions described, leads to complete or partial protection against intracerebral infection in about two-thirds of the cases. Dead virus, on the other hand, completely lacks any immunizing properties.

DISCUSSION

In analyzing the data reported in this paper, it seems likely that a dual mechanism of protection against poliomyelitis must be recognized in the monkey. Our experiments with adult *rhesus* monkeys and sub-adult chimpanzees leave no question that age alone conveys to the animal a certain degree of enhanced resistance, which in all probability is purely physiological. Attempts to reproduce in immature monkeys this relative state of mature insusceptibility by the adminis-

tration of single hormones, have led to success in the case of at least two internal secretions; *i.e.*, anterior pituitary hormone extracted from sheep glands and anterior pituitary-like principles from pregnancy urine of man. The enhancement of resistance, however, is only in rare instances sufficiently marked to protect against intracerebral infection, but manifests itself chiefly in the liberation of virucidal substances into the serum of the prepared animal. This intensification of serological activity is probably best explained by assuming an accelerated rise of preformed neutralizing substances from an imperceptible immature level to an experimentally recognizable, mature concentration under the influence of hormonal stimulation. Quite apart from this physiological enhancement of resistance, it is also clear that a proportionally higher degree of protection can be obtained by immunization with the specific virus. The two processes, although accomplishing similar results, are obviously totally dissimilar in mechanism.

When an attempt is made to correlate these experimental findings with the probable mechanism of susceptibility to poliomyelitis in man, no definite answer can be given. While it is premature to discuss here the debatable clinical evidence of the presence of endocrine stigmata in children attacked by poliomyelitis, some epidemiological inferences may be drawn with reasonable safety. There can be no question that immunization by the virus plays a certain part in populations where the disease is widely prevalent; and this immunization may conceivably take the form of responses to either latent or manifest antigenic stimuli. The first alternative was well demonstrated in a recent observation of Kramer's (24) who proved that children after contact with a case of poliomyelitis acquired virucidal properties in the serum, without developing any signs of the disease. This change in serum reaction, however, occurred only under conditions of very intimate contact implying an actual implantation of the virus, and failed to come about after casual contact, which, after all, represents the more common form of human relationship. The second possibility was experimentally substantiated when Trask and Paul (25) could show that children following an abortive attack of poliomyelitis developed a definite, though in most cases only transient, virucidal titer in the serum. If Faber (26) is correct in denying an extranervous phase of poliomyelitic infection, the frequent and re-

peated occurrence of so called abortive attacks among the population at large may again well be questioned.

It becomes therefore increasingly difficult to reconcile the extent and persistence of insusceptibility to the disease, particularly among the older age groups, with the known or suspected manifestations of immunization by the virus. The experimental facts recorded in this paper lend support to the opinion that the gradual increase in serological activity and the corresponding rise of resistance with age are largely due to a series of developmental changes in constitutional perfection, markedly influenced or even controlled by endocrine function (27) and the laws of heredity (28). The present status of the problem is probably best expressed by Armstrong (29): "There is certainly a specific immunity to poliomyelitis as there is to many other diseases, but the assumption that resistance to a virulent strain is always dependent upon a specific immunity gained through contact with feeble strains of that virus, is hardly an established fact."

CONCLUSIONS

1. Adult *rhesus* monkeys possess frequently a greater resistance to experimental infection with poliomyelitis virus than young monkeys as indicated by the prolongation of the incubation period and the content of feeble neutralizing substances in the normal serum.

2. Virucidal substances can be demonstrated in the normal serum of subadult chimpanzees.

3. Immature monkeys prepared with glandular anterior pituitary hormones and anterior pituitary-like principles from the urine of pregnant women are protected only in exceptional instances against intracerebral infection with poliomyelitis virus. However, the serum of such prepared animals frequently acquires the property of inactivating the virus *in vitro*.

4. Active immunization with live poliomyelitis virus renders the immunized monkeys resistant to intracerebral infection in a higher percentage of the cases than does preparation with hormone.

5. The relative merits of non-specific enhancement of resistance and of specifically acquired immunity are discussed in their relation to the probable mechanism of resistance to poliomyelitis in man.

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A STUDY ON BACTERIAL PROTEINS WITH SPECIAL CONSIDERATION OF GONOCOCCUS AND MENINGOCOCCUS*

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The isolation of "nucleoproteins" from microorganisms dates back to Stutzer's (1) chemical investigation of yeast in 1882; but their first identification in one of the common pathogens seems to have been the result of Aronson's (2) work on the diphtheria bacillus in 1900. Since that time a considerable literature has accumulated. When the mere presence of "nucleoproteins" in bacteria ceased to be a novelty, and their analysis proved too difficult for the classical techniques of biochemistry, interest shifted to their immunological behavior.

The few references which follow are to studies particularly pertinent to the problem in hand. Heidelberger and Avery (3, 4) prepared from pneumococci a "purified nucleoprotein" which was capable of engendering antibodies, and in its antigenic behavior exhibited species specificity but not type specificity. Lancefield (5) in 1925 studied the "nucleoproteins" of several strains of *Streptococcus viridans*, and subsequently (6) those obtained from hemolytic streptococci. Heidelberger and Kendall (7) fractionated the protein of a scarlatinal streptococcus. An exhaustive review of the published work on the tubercle bacillus is to be found in the recent book by Wells and Long (8).

The work reported below began as a study of the chemical and immunological properties of the protein and non-protein fractions obtained from gonococcus and, for purposes of comparison, from meningococcus. The unexpected finding of positive cross-reactions with immune serum to an unrelated species (Pneumococcus Type III) as reported in our preliminary communication (9) led to an extension of the investigation to include several other organisms. "Nucleoproteins" were therefore prepared from *Micrococcus catarrhalis*, *Staphylococcus aureus*, *Streptococcus hemolyticus*, and an R strain of pneumococcus.

Methods

The organisms were cultivated in ordinary 16 ounce medicine bottles on an agar medium[1] consisting of a tryptic digest of egg white (from which the heat-coagulable proteins had been removed), dextrose, and an appropriate mixture of salts. The 12 to 18 hour growth was removed with 0.9 per cent sodium chloride solution, filtered through a thin layer of cotton supported by gauze, separated by centrifugation, and washed thrice by alternate suspension in normal salt solution and centrifugation.

Organisms grown in liquid media were also employed in the preparation of bacterial nucleoprotein. In the case of gonococcus and meningococcus this medium contained the same ingredients as the solid, less the agar (11). For pneumococcus ordinary meat infusion broth was used. After incubation for 18 hours the organisms were recovered by centrifugation and treated in the same way as those grown on solid media. No differences were detected in the immunological behavior of the nucleoproteins prepared from a given organism grown on solid or in liquid medium. The former therefore became the routine method as it was the more efficient.

Extraction of the so called "nucleoprotein" from the bodies of the organisms was accomplished by the several methods described below. The procedures varied from rather drastic chemical treatment to simple mechanical disintegration of the cellular structure. The method employed at the beginning of this study was that which Lancefield had found satisfactory for the preparation of her streptococcal nucleoproteins, briefly, extraction with N/100 sodium hydroxide. It was soon found, however, that alkali in considerably lower concentrations would extract the proteins of gonococcus and meningococcus, and the method was accordingly modified to the one described under Section 6 below. But when certain unexpected cross-reactions were encountered, consideration was given to the possibility that alkali even so dilute might have denatured the proteins. For purposes of control, therefore, preparations were made by such innocuous, though relatively inefficient methods as to rule out this possibility.

Stained smears of the organisms were examined microscopically to compare the effects of the various treatments on their morphology. Only insignificant changes were observed in the contour of the Gram-negative cocci, though the cellular content of stainable material was reduced roughly in proportion to the quantity of alkali to which they had been exposed; *i.e.*, to their loss of protein by extraction.

1. Extraction with Distilled Water.—The well packed bacteria were suspended in 3 to 4 volumes of cold distilled water and allowed to stand for 12 hours at 4–8°C. The yield from this extraction was very small, as was to be expected, since the true nucleoproteins and many other protoplasmic constituents are insoluble in water.

2. Maceration with Sodium Chloride.—To 20 cc. of fresh, moist organisms, well packed by centrifugation, were added 22.5 gm. of fine crystals of sodium chloride,

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[1] Described as the control medium in the paper by Miller, Hastings, and Castles (10).

and the mixture was vigorously ground in a mortar from time to time during 1 day. After standing overnight at room temperature, 2.5 liters of distilled water were added, a quantity sufficient to reduce the concentration of sodium chloride to about 0.9 per cent. It was presumed that the cells might take up sufficient salt during their maceration to cause their disruption when the distilled water was added. This method was somewhat more efficient than the first. The yield was further increased by the addition of sufficient solid sodium carbonate to the salt crystals to make a final concentration of 0.27-0.5 per cent.

3. *Grinding in the Dry State.*—Fresh organisms were spread in a thin layer on a watch-glass and dried *in vacuo* over sulfuric acid. They were then ground for 72 hours in a rotary ball mill and subsequently extracted with distilled water.

4. *Freezing and Thawing.*—Moist, packed organisms were rapidly frozen and thawed 4 times, then suspended in water. Microscopical examination showed most of the cells to be still intact.

5. *Surface Tension Depressants.*—Attempts were made to effect lysis of the organisms by purified solutions of sodium oleate, sodium ricinoleate, and sodium taurocholate. With the exception of pneumococcus, which was promptly dissolved by each of these, the result was no better than that accomplished by simple alkalinization to the same degree. (See Section 6 below.) Difficulties were encountered in preparing the proteins from these extracts as traces of the soaps could not be removed and gave false precipitin reactions.

6. *Treatment with Dilute Alkali.*—Organisms were suspended in 10-15 volumes of cold distilled water containing a trace of phenol red as indicator. Sufficient sodium hydroxide was added to bring the reaction to pH 7.6-7.8. The suspension was kept in the refrigerator at 4-8°C. for 12-18 hours, during which time the reaction was maintained at pH 7.6-7.8 by the addition, whenever necessary, of sodium hydroxide. It was found that a batch of 20 cc. of packed organisms, suspended in 2.5 liters of water, usually required a total of about 25 cc. of $N/100$ sodium hydroxide. This method was found to be the most satisfactory and was employed in preparing the proteins from the three Gram-negatives and pneumococci used in the experiments which follow. It was chosen because it was more efficient than the foregoing and yielded a product indistinguishable immunologically from those obtained by simpler means. In the case of the staphylococcus and streptococcus, satisfactory extraction was accomplished only in $N/100$ NaOH, so that method was necessary in the preparation of their proteins.

The products obtained by these various methods of extraction are doubtless of different composition, since the components of so called "bacterial nucleoprotein" (albumin, globulin, mucin, nucleoprotein, etc.) differ in their respective solubilities in water, dilute alkali, and normal saline; but the variation in composition was regarded as quantitative rather than qualitative because all of the preparations from a given organism gave the same immunological reactions.

Preparation of the Protein.—The extract was freed by centrifugation from insoluble matter in suspension, and to it was slowly added, with constant stirring, dilute acetic acid, in the least quantity required to give a maximum precipitate.

After the heavy precipitate had settled, the supernatant liquid was decanted and used in the preparation of the carbohydrate described in the following paper (12).

The precipitate was dissolved in sodium hydroxide solution, at pH of about 7.8, reprecipitated with acid, and centrifuged. This precipitation was repeated once more. Then the protein was dissolved and dialyzed in bags of cellophane (No. 600) against distilled water for several days. During dialysis the protein solution was covered by a protecting layer of toluene. The dialyzed solution was evaporated to dryness in an air current at 56°C. and preserved in the dry state.

A later paper will report our findings on the peculiar nature and immunological effect of some of the individual proteins obtained by fractionating these protein mixtures.

Properties

The bacterial protein used in these experiments is designated "nucleoprotein" (in accordance with common usage), although it is admittedly a mixture of acid-precipitable cellular proteins, including albumins, globulins, etc., as well as the true nucleoproteins. All of the preparations were partially soluble in distilled water and entirely soluble in a solution made alkaline, pH 7.6, with sodium hydroxide. They gave all the usual protein reactions; *e.g.*, biuret, xanthoproteic, Millon, Hopkins-Cole, sulfosalicylic acid and phosphotungstic acid tests. A positive Molisch test on each of them indicated the presence of a carbohydrate radical.

Toxicity for Laboratory Animals.—Intravenous injection of saline solution of gonococcal or meningococcal nucleoprotein in any considerable amounts proved lethal to rabbits. A series of experiments was therefore performed to compare the relative toxicity of these two organisms with their respective nucleoproteins. In the interest of economy of material, 20 gm. mice were used as the test animal and intraperitoneal injection as the method of administration.

Freshly grown organisms were spread on a watch-crystal, desiccated *in vacuo*, and taken up in sufficient saline to make a 0.5 per cent suspension on a basis of dry weight. In the case of meningococcus the suspension was heated at 56°C. for an hour. The nucleoproteins were likewise used in 0.5 per cent solutions. The concentration of protein in the bacterial suspensions was therefore somewhat less than in the nucleoprotein solutions, but, since no quantitative determinations were made of the organisms' actual content of this constituent, more accurate equivalence was impossible. The results were found to be significant, this small discrepancy notwithstanding.

Several experiments indicated the minimum lethal dose of gonococci for white mice to be between 2.5 and 5.0 mg. and that of the nucleoprotein to be only slightly greater. As the error inherent in such a method of biological assay can be corrected only by the use of numbers of animals great enough to be treated statistically, these figures must be regarded as close approximations. The experiment in Table I shows, however, that mice succumbed more quickly to injections of whole organisms than to equal quantities of nucleoprotein of the same strain.

TABLE I

Relative Toxicity for Mice of Gonococci (Intact Organisms) and of Nucleoprotein Prepared from the Same Strain

Material and dose		No. of mice injected	No. of mice surviving at the end of				Total dead
			12 hrs.	18 hrs.	36 hrs.	60 hrs.	
	mg.						
Organisms............	1.25	3	3	2	2	2	1
Nucleoprotein........	1.25	3	3	3	3	3	0
Organisms............	2.5	5	3	3	1	0	5
Nucleoprotein........	2.5	5	5	5	4	4	1
Organisms............	5.0	5	3	0	0	0	5
Nucleoprotein........	5.0	5	5	4	0	0	5
Organisms............	10.0	10	7	1	0	0	10
Nucleoprotein........	10.0	10	9	3	2	2	8

Table II gives the results of a similar experiment designed to compare the toxicity of meningococcus with that of nucleoprotein prepared from the same strain. It indicates that intact meningococci were somewhat more toxic than their nucleoprotein, the difference here being more apparent than in the case of gonococcus. That the heating of the bacterial suspension did not appreciably reduce its toxicity can be confidently assumed, for this property has been found to be remarkably resistant to heat. A number of experiments (13) have, in fact, shown that prolonged exposure to much higher temperatures is necessary to diminish perceptibly the lethal action of meningococci for mice. In this connection should be noted the work of Branham and Lillie (14)

on experimental meningitis in guinea pigs. They found that intra-cisternal inoculations of boiled and living meningococci produced essentially identical clinical and histopathological pictures.

Effect of Defatting.—Extraction with acetone and ether in the cold did not appreciably alter the toxicity for mice, nor the antigenic properties, as measured by precipitin reactions, of gonococci and meningococci (whole organisms) and their nucleoproteins.

Allergic Reactions with Nucleoproteins.—Rabbits rendered hypersensitive to gonococci and meningococci by the subcutaneous implanta-

TABLE II

Relative Toxicity for Mice of Meningococci and of Nucleoprotein Prepared from the Same Strain

Material and dose		No. of mice injected	No. of mice surviving at the end of				Total dead
			12 hrs.	18 hrs.	36 hrs.	60 hrs.	
	mg.						
Organisms...........	0.62	5	4	4	4	4	1
Organisms...........	1.25	5	3	3	2	2	3
Nucleoprotein........	1.25	5	5	5	5	5	0
Organisms...........	2.5	5	3	3	2	1	4
Nucleoprotein........	2.5	5	3	3	2	2	3
Organisms...........	5.0	5	4	2	0	0	5
Nucleoprotein........	5.0	5	4	3	3	2	3
Organisms...........	10.0	10	1	0	0	0	10
Nucleoprotein........	10.0	10	7	4	3	2	8

tion of masses of infected agar (see Miller and Castles (15)) were found to be equally allergic to their nucleoproteins. The reactions were always of the delayed type. When gonococcal nucleoprotein was used as the sensitizing ingredient in the subcutaneous agar foci, the rabbits developed an even higher degree of allergy than to viable gonococci. The nature of the inflammatory reaction about these foci was not studied. No difference was detected in the specificity of the hypersensitiveness evolved by nucleoprotein and by the organisms themselves.

Immunological Reactions

Preparation of Immune Sera.—Antisera were prepared from the nucleoproteins of gonococcus, meningococcus, and *M. catarrhalis.* Snuffle-free rabbits weighing from 4-6 pounds were used. They received by intravenous injections at 4 to 5 day intervals, a 1 per cent solution of the protein in saline adjusted to pH 7.6–7.8. The doses were increased progressively (1, 2, 4, 6, 8, and 10 cc.), and the animals were bled 5 days after the last injection. It should be noted that this slight alkalinization seemed to diminish somewhat the toxicity of the protein.

Antibacterial sera were also prepared, in the case of gonococcus, meningococcus, *M. catarrhalis*, and an R pneumococcus, by intravenous immunization of rabbits with intact organisms suspended in saline. These immunizations were effected more slowly as the greater toxicity of the inocula demanded greater caution.

Precipitin Reactions.—The precipitin tests were made by the contact method in which serum diluted 1:3 with 0.9 per cent sodium chloride solution was placed under progressive 10-fold dilutions of the protein in normal saline, usually beginning at 1:1000. The titers of the antisera were designated as the highest dilution of antigen which showed definite clouding at or near the intersurface of the two liquids when examined in bright illumination against a black background. This method eliminates the variability of precipitation commonly known as inhibition zones. The tests were made in small vials (26 x 5 mm.) which had been thoroughly cleansed with hot chromic acid cleaning solution and many applications of distilled water.

Results.—The results of a series of precipitin tests on nucleoprotein preparations from five strains of gonococcus with five representative immune sera are given in Table III. Two of the sera were obtained by immunization with whole organisms (individual strains) and two with the nucleoproteins of those same strains, while the fifth was prepared by immunization with a mixture of several strains, including the foregoing. It will be noted that the titers vary from 1:10,000 to 1:1,000,000, but that the sera did not react to their homologous proteins in unusually high dilutions. Nor did the polyvalent serum show differences from the rest. The titers of the antiprotein sera averaged about the same as the antibacterial.

Table IV presents the results of precipitin reactions in which the nucleoproteins of 6 organisms served as antigens, and the following as precipitating sera: antigonococcus, antimeningococcus, and anti-*catarrhalis* sera obtained by the immunization of rabbits, and the three antipneumococcus sera commonly used for typing pneumococci. Gonococcal nucleoprotein was precipitated by antimeningococcus

serum and by antipneumococcus sera of all three types, but not by anti-*catarrhalis* serum. The latter, nevertheless, gave a positive reaction with meningococcal nucleoprotein, which was precipitated by only Type III of the antipneumococcus sera. Positive reactions were

TABLE III

Precipitin Reactions with Gonococcal Nucleoproteins Prepared from Different Strains

Nucleo-protein strain	Sera prepared by immunizing rabbits to									
	Strain 1				Strain M_6B_2				Organisms of several strains	
	Nucleoprotein		Organisms		Nucleoprotein		Organisms			
1	+++	+	+++	++	+++	+++	+++	+++	+++	+++
3	+++	+	+++	+	+++	++	+++	++	+++	+
5	+++	++	+++	++	+++	++	+++	++	+++	++
M_6B_2	+++	+	+++	++	+++	++	+++	++	+++	++
10	+++	++	+++	+++	+++	++	+++	+++	+++	++

In this and the following tables, the plus marks indicate dilutions of precipitinogen as multiples of 10; thus: +++ = 1:1000; +++ + = 1:10,000, etc. — = negative in dilution of 1:1000.

TABLE IV

Precipitin Tests with Nucleoproteins Showing Non-Specific Cross-Reactions

Nucleoproteins prepared from	Sera							
	Anti-gonococcus		Anti-meningococcus		Anti-catarrhalis		Antipneumococcus		
							Type I	Type II	Type III
Gonococcus	+++	++	+++		—		+++	+++	+++ +
Meningococcus	+++	++	+++	+++	+++	+	—	—	+++ +
M. catarrhalis	+++		+++		+++	+	—	—	+++
R pneumococcus	+++	+	—		—		+++	+++	+++ +
Strep. hemolyticus	—		—		—		—	—	+++
Staph. aureus	—		—		—		—	—	+++

obtained with the nucleoprotein prepared from an R strain of pneumococcus and antigonococcus, but not with antimeningococcus nor anti-*catarrhalis* sera.

Effect of Tryptic Digestion of the Nucleoprotein.—Gonococcal and meningococcal nucleoproteins were subjected to digestion by trypsin

and then tested for certain of the cross-reactions, together with nucleo-protein solutions which had been alkalinized (to pH 7.8) and heated (to 56°C.) the same as the digests. The results presented in Table V show that both nucleoproteins failed after digestion to react with Anti-pneumococcus Serum Type III, but continued to react with sera to closely related organisms (the other *Neisseriae*).

Precipitin Reactions with Other Sera.—Both gonococcal and menin-gococcal nucleoproteins failed to react when tested with a variety of

TABLE V

Precipitin Reactions with Tryptic Digests of Gonococcal and Meningococcal Nucleoproteins

Precipitinogen	Immune sera			
	Anti-gonococcus	Anti-meningococcus	Anti-catarrhalis	Antipneu-mococcus Type III
Gonococcal nucleoprotein	+++ +	+++	⎧Not	+++ +
Gonococcal nucleoprotein after tryptic digestion	+++ +	+++	⎩made	−
Meningococcal nucleoprotein	+++	+++ ++	+++ +	+++ +
Meningococcal nucleoprotein after tryptic digestion	+++	+++ ++	+++ +	−

other sera, which included typhoid, paratyphoid A and B, dysentery, anthrax, and human serum containing a high titer of agglutinins for *B. melitensis.*

DISCUSSION

As has been mentioned, the term "nucleoprotein" is employed in accordance with the usage of bacteriologists rather than of chemists, for it designates not a chemical entity but a mixture of substances in-cluding the true nucleoproteins. The present report concerns certain properties of the nucleoproteins of gonococcus and meningococcus, and for purposes of comparison, of *M. catarrhalis*, R pneumococcus, *Streptococcus hemolyticus*, and *Staphylococcus aureus.*

Both gonococcal and meningococcal nucleoproteins engendered pre-cipitins in rabbits which were indistinguishable by cross-reactions with

other proteins from the precipitins engendered by the intact organisms of these two species. In other words, the antigenic factors responsible for these cross-reactions were neither destroyed nor created by the chemical treatment involved in their preparation, which, for most of our material, included dilute alkali. In the case of gonococcus, the harmlessness of our standard method was also checked by making several small lots which were extracted by simple physical means without the use of alkali. These preparations, with the exception of those subjected to the action of soaps of fatty acids, showed no differences from the others which could be detected by the immunological technique employed.

The cross-reactions between gonococcal nucleoprotein and antipneumococcus serum of all three types, as well as those between meningococcal and *catarrhalis* nucleoproteins and Type III serum, are, therefore, regarded as heterogenetic reactions. It is interesting that the antigenic factor responsible for this reaction was destroyed by tryptic digestion, although those responsible for the reactions with their homologous sera and for cross-reactions with those to closely related species (the other Gram-negative diplococci) were not disturbed by such treatment.

In the phenomenon of bacterial allergy nucleoproteins were found to play the same rôle as viable organisms; for the nucleoproteins of gonococci and meningococci evoked, in hypersensitive rabbits, quite as strongly positive cutaneous reactions as did the organisms themselves. And in its ability to induce the allergic state, gonococcal nucleoprotein (the only one so studied) seemed to be even more effective than living organisms. This observation may be explained by its solubility in the body fluids, hence its easier availability to the tissues of the animal. The specificity of the hypersensitiveness so induced and of the cutaneous reactions to nucleoprotein was no sharper than to intact organisms.

The toxicity of both gonococcal and meningococcal nucleoproteins, as determined by their lethal action in mice, was found to be only slightly less than that of the intact organisms of those two species. This indicates that most, if not all, of the toxic property of these organisms is due to some constituent of the fraction designated "nucleoprotein," which is regarded as existing naturally within the

body of the bacterial cell. We have been unable, in fact, to demonstrate any "toxin" in cultures of these organisms either in liquid or on solid media until they had reached an age when some members of the population had begun to disintegrate, or unless the hydrogen ion concentration in the surrounding liquid was such as to favor the extraction of nucleoprotein from the cells.

Investigation of the toxicity as well as the immunological behavior of the products of further fractionation of these proteins is now in progress.

SUMMARY AND CONCLUSIONS

Methods of preparation and certain properties of the "nucleoproteins" of the following organisms are described: gonococcus, meningococcus, *Micrococcus catarrhalis*, R pneumococcus, *Streptococcus hemolyticus*, *Staphylococcus aureus*. No essential differences between the nucleoproteins and the intact cells of gonococcus and meningococcus were observed in their ability to engender immune substances (precipitins), to induce bacterial allergy in rabbits, or to elicit cutaneous reactions (of the delayed type) in rabbits rendered hypersensitive to these organisms. Measured by their lethal action in mice, the toxicity of gonococcal and meningococcal nucleoproteins was found to be but slightly less than that of the intact cells. It seems probable, therefore, that the toxic action of these organisms is due, chiefly or entirely, to some constituent of the nucleoprotein fraction. Extraction with acetone and ether in the cold did not reduce appreciably the toxicity of these organisms and their nucleoproteins, nor alter their immunological behavior.

Cross-precipitin reactions suggested that gonococcal nucleoprotein contains an antigenic factor in common with the non-encapsulated pneumococcus cell, and meningococcal nucleoprotein one in common with the capsular material of Pneumococcus Type III. Tryptic digestion destroys these antigenic factors, but not those responsible for the cross-reactions within the genus *Neisseria*.

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THE CARBOHYDRATES OF GONOCOCCUS AND MENINGOCOCCUS

I. The Alcohol-Precipitable Fraction[*]

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As early as 1874 Scheibler (1) studied a polysaccharide isolated from the gum of *Streptococcus* (*Leuconostoc*) *mesenterioides*, an organism of some economic importance to the manufacturers of sugar; but little attention was given to the carbohydrates of pathogenic bacteria until Toenniessen's (2) work on Friedländer's bacillus. Within the past decade and a half, however, advance in this field has been accelerated by the use of immunological methods as an adjunct to the usual techniques of organic chemistry. Zinsser and his coworkers (3, 4) prepared from several organisms residue antigens which were almost protein-free, reacted by precipitation with homologous immune sera, but failed to elicit antibody production in animals. Avery and Heidelberger (5, 6) carried out a most exhaustive and fruitful investigation of the carbohydrates of pneumococcus. They obtained from Types II and III products which were nitrogen-free and chemically distinct from each other and from that of Type I which contained some nitrogen, presumably as an integral part of its molecule. They were non-antigenic in rabbits, but reacted specifically in extremely high dilutions with homologous antisera. From a rough strain of pneumococcus Tillett and Francis (7, 8) obtained a non-type-specific carbohydrate, designated the C fraction. Recently Wadsworth and Brown (9) have reported the isolation of type-specific substances of a carbohydrate nature from each of the fixed types of pneumococci which they regard as different from the polysaccharides of Avery and Heidelberger and also from the C fraction of Tillett and Francis.

The preparation of carbohydrate fractions from a number of other organisms has been accomplished, but only the work of Casper (10) on gonococcus, and that of Zozaya and Wood (11) and of Webster and Rake (12) on meningococcus relates to the present study.

This study has been carried along parallel with the work on the nucleoproteins reported in the preceding paper (13). It began, therefore, with the preparation and investigation of carbohydrate fractions from gonococcus and meningococcus, the two organisms of special interest to us. Subsequently *Micrococcus catarrhalis*, R pneumococcus, *Streptococcus hemolyticus*, and *Staphylococcus aureus* were added for purposes of comparison.

Methods

The organisms were grown and extracted by the methods described in the preceding paper (13). The supernatant liquid from the acetic acid precipitation of the proteins was filtered, neutralized with sodium hydroxide, and evaporated to small bulk (each liter to 25 cc.) at 56°C., in an air current. This concentrated solution was then made slightly acid and placed in boiling water for 7 minutes to remove the remaining proteins by heat coagulation. The solution was then filtered and added to 7 to 8 volumes of 95 per cent ethyl alcohol. The resulting precipitate was allowed to remain in the alcohol for several hours to facilitate its denaturing action on any traces of protein still present. The supernatant alcoholic solution was decanted, filtered, and evaporated to dryness. The residue from this evaporated alcoholic solution was found to be protein-free by all the protein tests to which it was subjected. Aside from the sodium acetate (from the acetic acid and sodium hydroxide used) it consisted principally of carbohydrate, a fraction which will be the subject of a later report. The alcoholic precipitate contained the fraction which primarily concerns us here. It also proved to be chiefly polysaccharide, but may have included traces of amino acids and any alcohol-insoluble, non-heat-coagulable, non-acid-precipitable proteins. It was centrifuged and drained free from excess liquid. While still damp it was dissolved in water, then twice reprecipitated from 80 per cent alcohol of first slightly alkaline and then slightly acid reaction. Finally the aqueous solution was dialyzed in a cellophane bag against distilled water.

Not all of the preparations were carried through the final step of repeated precipitation and dialysis, because considerable loss in carbohydrate resulted. It should be noted, however, that in immunological behavior such preparations did not differ from those which had been more highly purified.

Physical and Chemical Properties

In the dry state the carbohydrates prepared from all of the organisms were light yellow in color. They were entirely soluble in distilled water

and in 0.9 per cent sodium chloride solution. When ethyl alcohol was added to an aqueous solution precipitation began at an alcoholic concentration of about 40 per cent and increased with each addition of alcohol until the concentration reached 80 per cent. They seemed to be resistant to the action of weak acids and alkalies. They were only partially dialyzable; that is to say, a detectable loss occurred after 10 days dialysis in a bag of cellophane (No. 600) against distilled water. Possibly products of a slow hydrolysis of the polysaccharide were lost (escaped through the membrane).

The tests for protein, e.g. biuret and xanthoproteic, were negative, although carbohydrate reactions, as the Molisch test, were strongly positive. The solutions retained in the cellophane bag after dialysis gave negative Benedict-Fehling reduction tests. However, this test was positive when performed on hydrolysates obtained by boiling the carbohydrates with 2 per cent hydrochloric acid.

The preparations most extensively studied were those of gonococcus and meningococcus. Negative ninhydrin reactions indicated the absence of an amino acid impurity in appreciable quantity. The Millon and Hopkins-Cole tests were also negative. Negative orcinol, phloroglucinol, and resorcinol reactions indicated the absence of pentose and ketose radicals. The nitrogen content of the gonococcal polysaccharide was found by Kjeldahl micro determination to be 4.2 per cent. An aqueous solution of this polysaccharide was optically inactive. The nitrogen content of the meningococcal polysaccharide was 3.7 per cent.

Toxicity for Laboratory Animals.—Both the gonococcal and meningococcal carbohydrates were non-toxic for rabbits and mice. The former received the material intravenously, the latter intraperitoneally, without evidence of deleterious effect.

Antigenicity of the Carbohydrate Preparations.—The sera of rabbits which had been repeatedly injected intravenously with carbohydrate fractions prepared from gonococci and from meningococci contained no antibodies demonstrable by the precipitin reaction. An additional attempt was made, patterned after the method employed so successfully by Landsteiner with his alcohol-soluble haptenes, in which the rabbits were injected intravenously with gonococcal carbohydrate mixed with pig serum. These animals developed high titers of anti-pig serum precipitins but none at all for the carbohydrate.

Cutaneous Reactions with Gonococcal Carbohydrate.—In rabbits rendered hypersensitive by the implantation of agar foci containing either gonococci or meningococci (14), allergic reactions of the delayed type were elicited by the intracutaneous injection of 0.1 cc. of a 1:1000 solution of gonococcal carbohydrate. No reaction followed intracutaneous injection into normal (snuffle-free) rabbits.

Immunological Reactions.—Precipitin reactions were made in the same fashion and with the same sera as those described in the preceding paper. A summary of representative tests is given in Table I. Cross-reactions occurred between the carbohydrates of the gonococcus and meningococcus and their respective antisera, a result which was to be

TABLE I

Precipitin Reactions with Carbohydrate Preparations

Carbohydrates prepared from	Rabbit sera prepared by immunization with			Commercial sera	
	Gonococcus	Meningococcus	M. catarrhalis	Antimeningo-coccus	Antipneumo-coccus Type III
Gonococcus........	+++ ++	+++	—	+++ +++	+++ +++
Meningococcus.....	+++ ++	+++ ++	—'	+++ +++	+++ ++
M. catarrhalis......	+++ +	—	+++ +	+++ +	+++
R pneumococcus ...	+++ +	—	—	+++ +	+++ +
Strep. hemolyticus...	—	—	—	—	+++
Staph. aureus.......	—	—	—	—	—

The plus marks indicate dilutions of precipitinogen as multiples of 10; thus: +++ = 1:1000; +++ + = 1:10,000; etc. — = negative in dilution of 1:1000.

expected. The carbohydrate of *M. catarrhalis* was precipitated by antigonococcal serum, while the anti-*catarrhalis* serum failed to react with the polysaccharides of either of those organisms. An inconsistency is to be noted in the case of *M. catarrhalis* which reacted with commercial antimeningococcal serum but not with that prepared in the laboratory by the immunization of rabbits. This may be explained by the fact that the titer of the rabbit serum was lower (only 1:100,000 for the carbohydrate of the homologous organism). The immunizations of the rabbits were purposely carried only to the point at which they yielded sera of workable titers in the hope that their specificity might be sharper.

Sera prepared by the immunization of rabbits with the "nucleo-proteins" of gonococcus and meningococcus gave the same reactions as the antibacterial sera, though usually in somewhat lower titer.

Antipneumococcus Serum Type III reacted with the carbohydrates of gonococcus and meningococcus in dilutions of 1:1,000,000 and 1:100,000 respectively, and with the carbohydrates of *M. catarrhalis* and hemolytic streptococcus in dilutions of 1:1000. Carbohydrates isolated from an R pneumococcus (one prepared by the method herein described and one by the method of Tillett and Francis (7)) reacted in dilutions of 1:10,000 not only with antipneumococcus serum of all three types but with antimeningococcus and antigonococcus serum as well.

Strain Specificity.—Carbohydrate fractions prepared from 6 strains of gonococcus employed in precipitin reactions with immune sera to 5 strains of that organism showed no evidence of strain specificity.

Reactions with Other Sera.—Gonococcal and meningococcal poly-saccharides gave negative precipitin reactions with commercial sera to the following organisms: typhoid, paratyphoid A and B, dysentery and anthrax bacilli, and several varieties of streptococci. Negative results were also obtained with diphtheria and scarlet fever antitoxins.

DISCUSSION

Since the classical investigations of Landsteiner, evidence has ac-cumulated to support his original conception of the nature of anti-genic specificity. It is now generally accepted that the specificity of pure proteins may reside in individual radicals, while that of highly complex antigens may depend upon components of relatively simple chemical composition. These components in the case of a number of bacterial antigens have been found to be polysaccharides, and the carbohydrates herein described are also so regarded. No evidence was obtained to preclude the assumption that they exist *in vivo* as part of a glycoprotein. As noted in the preceding paper, the "nucleoproteins" described gave strongly positive Molisch reactions. Furthermore sera prepared by immunization with gonococcal and meningococcal "nucleoproteins" reacted with the protein-free carbohydrates of those organisms.

Webster and Rake (12) in a preliminary communication report the

isolation from meningococcus of a type-specific as well as a non-specific polysaccharide. Until the publication of their methods we cannot compare our work with theirs, but the conjecture seems obvious that the carbohydrates herein described correspond to their non-specific polysaccharide.[1] It should be noted that our preparations were made from thrice washed organisms, and it is quite possible that a more specific constituent was lost in this process.

As it has been impossible to demonstrate capsules on gonococci or meningococci, the carbohydrates described have been regarded as somatic in origin, and therefore analogous to the C fraction isolated by Tillett and Francis (7) from rough pneumococcus.

The cross-reactions between the carbohydrates of gonococcus and meningococcus and antisera to those two organisms were to be expected from their biological similarity. So were the reactions of *catarrhalis* carbohydrate, which were positive with antigonococcus and negative with antimeningococcus serum, and likewise the failure of anti-*catarrhalis* serum to react with the carbohydrates of the other two *Neisseriae*. For these findings are in harmony with the relationship suggested by the work of Miller and Castles (14) on the allergic cutaneous reaction to these organisms.

The precipitin reactions of Antipneumococcus Serum Type III with the carbohydrates of gonococcus and meningococcus, however, were quite unexpected. They are doubtless analogous to the several other hetero-antigenic relationships among bacteria which have been reported; *e.g.*, between Strain E of Friedländer's bacillus and Type II pneumococcus reported by Avery, Heidelberger, and Goebel (15), and meningococcus, *B. anthracis*, *B. subtilis*, *B. proteus*, and *B. mesentericus* by Zozaya (16). The latter subsequently (17) qualified his statements about these cross-reactions, in the light of his observation that bacteria grown on solid media may adsorb enough agar to engender antibodies to it. We, ourselves, had considered this possibility (suggested by Furth and Landsteiner (18)), as well as the possibility that traces of undigested egg white which had failed to precipitate in the preparation of our media might adhere to the bodies of the organisms and cause

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[1] Since the presentation of this manuscript for publication, the study referred to has been reported under the authorship of Rake and Scherp (20).

the hetero-reactions which we encountered. Control tests were accordingly run with the agar medium as precipitinogen; but they were all negative and therefore received no mention in our preliminary communication (19). As an additional check on the egg white digest medium as a source of non-specific reactions, rabbits were immunized to egg white, but their sera failed to react with our carbohydrate and nucleoprotein preparations, although they contained anti-egg white precipitins in very high titers. Since the publication of Zozaya's paper (17) these control tests have been repeated. With the following exceptions the results duplicated those obtained 2 years ago. These exceptions warrant description. Agar, in dilution of 1:1000, when superimposed on certain of the commercial antimeningococcus and antipneumococcus sera,[2] showed at the end of an hour the formation of a translucent flocculus above the interface of the two liquids. It was much greater in bulk than the heaviest of precipitin rings, but was unlike one in consistency, and showed no tendency to settle, even to the upper surface of the serum. The highest dilution of agar which produced this reaction was 1:10,000 with antimeningococcus, and 1:1000 with certain antipneumococcus sera, irrespective of type. Whether or not this phenomenon should be regarded as a precipitin reaction is being left for the present an open question.

More pertinent to the problem at hand are the negative control tests with our rabbit immune sera (on egg white, media, and agar), and the fact that carbohydrates prepared from gonococci and meningococci grown in liquid media gave the same non-specific cross-reactions as those prepared from organisms grown on agar medium. It seems highly improbable, therefore, that the cross-reactions herein described are due to agar adsorbed by the bacteria during their cultivation.

As has been noted, the carbohydrates described above are precipitable by alcohol from bacterial extracts after the removal of their "nucleoproteins." In the case of gonococcus and meningococcus two other fractions are at present under investigation: an alcohol-soluble carbohydrate and a bacterial residue of difficult solubility which seems also to be composed of, or to be rich in carbohydrate.

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[2] These sera were from different lots, as those used 2 years ago were no longer available.

SUMMARY

The alcohol-insoluble polysaccharides of gonococcus and meningo-coccus were found to contain 4.2 and 3.7 per cent nitrogen respectively, to be protein-free by chemical test, to reduce Fehling-Benedict solution only after hydrolysis. They were non-toxic for rabbits and mice, and failed to engender antibodies (precipitins) in rabbits. They produced no cutaneous reactions in normal, snuffle-free rabbits, but caused typical allergic reactions of the delayed type in rabbits rendered hypersensitive to these organisms. Both carbohydrates reacted in high dilution with Antipneumococcus Serum Type III. For comparison, carbohydrates were prepared also from *Micrococcus catarrhalis*, *Streptococcus hemolyticus*, *Staphylococcus aureus*, and a rough strain of pneumococcus.

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THE TOXIC PROPERTIES OF SERUM EXTRACTS OF HEMOLYTIC STREPTOCOCCI

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Although much work has been done in the search for a toxin of *Streptococcus hemolyticus* having lethal properties, no such toxin has, to date, been discovered. It is true that sterile filtrates of streptococcus cultures contain certain toxins, hemotoxins (1), leucocidins (2), Dick toxins (3), but none of these filtrates has been shown to be toxic for animals except in relatively enormous doses (4). Yet from the symptoms and pathology of streptococcus infections in man and in animals, there is every reason to believe that, *in vivo*, virulent strains of the streptococcus do produce a toxin or toxins with marked toxic properties which cause death. It was in the attempt to produce, *in vitro*, such a toxin or toxins that this work was undertaken.

In the course of some work on the production of powerful streptococcal hemotoxin, it was discovered that under certain definite conditions, the organisms themselves, if centrifuged down from broth cultures and taken up in the same volume of physiological salt solution or broth, were very hemolytic in minute amounts. That this hemolytic action had nothing to do with the growth of the organism in the red cell suspension was proved by plating the preparations, before, during, and after incubation and finding that there was no increase or only a slight increase of streptococci during the incubation. From these experiments it seemed probable that the streptococcal hemotoxin was bound to the surfaces of the organisms whence it was given off directly to the red cells.

Furthermore, the powerful hemotoxic effect of these streptococcus suspensions was obtained only from young cultures of 13 to 16 hours

growth. The hemotoxic content of suspensions of older cultures diminished directly with the age of the culture until practically no hemotoxin was demonstrable in suspensions of 2 days growth. Heating hemolytic suspensions to 56°C. for 20 minutes completely destroyed their hemotoxic properties.

It was next discovered that these hemotoxic streptococcus suspensions were extremely toxic for mice, causing death within 2 hours when injected intravenously. This lethal action of the suspensions appeared to be bound up in some way with their hemolytic content because suspensions of old cultures containing little hemotoxin or hemotoxic suspensions heated to 56°C. for 20 minutes were non-toxic for these animals.

In this work with streptococcus suspensions, the important question to be answered seemed to be what caused the death of these mice? Was death due to the hemotoxin *per se*, to some other toxic substance present with the hemotoxin, or to both, or was death brought about by some physical property of the preparation not present in suspensions of old organisms nor in heated suspensions? If death was caused by some toxic substance or substances attached to the surface of the organisms, it seemed possible that the toxic substance or substances might be removed by extracting streptococci with various materials. This we were successful in doing. By treating streptococci with serum, we obtained toxic extracts which were hemolytic and which, in relatively small amounts, killed mice. The following report deals with the methods used in the preparation of these toxic extracts and the experiments made to determine their nature.

Methods

Preparation of Media.—The medium used in this work which gave the best results was prepared as follows: 2 pounds of chopped veal in 850 cc. of distilled water were allowed to stand in the ice box for 2 or 3 days. Then in the meat juice recovered by squeezing through cheese-cloth, 40 gm. of Witte peptone were dissolved by heating the mixture to 45°C. for 20 minutes. After this the meat extract adjusted to a pH of 4.8 with N HCl, was poured into a 1 liter flask, tightly corked, and was boiled in a water bath for 30 minutes. Then to the clear meat extract supernatant recovered by centrifuging were added a sterile Berkefeld filtrate of 2 gm. of $NaHCO_3$ and 1 gm. of $Na_2HPO_4 \cdot 12H_2O$ dissolved in 100 cc. of distilled water. The whole preparation was next brought up to a pH of 8.1 with N NaOH, heated in a water bath at 70°C. for 15 minutes, cooled, and centri-

fuged. Finally the clear supernatant obtained by centrifuging was dispensed into centrifuge tubes 6 x 1 inch, 35 to 40 cc. to each tube, chilled, and heavy vaseline seals were added.

It is noted that in the preparation of this medium, the meat extract is heated at a high temperature only when adjusted to a low pH and that all filtration except in the instance of the buffers, is excluded.

When proper precautions are taken in the handling of the materials, the medium described above is sterile. In twenty-three consecutive batches no contaminations were encountered.

Source of Cultures.—Only 1 strain of *Streptococcus hemolyticus* was used in this work. This was an erysipelas strain obtained from Dr. Ada Clarke of the Department of Bacteriology, College of Physicians and Surgeons.

Sera Used.—Sheep, horse, and rabbit sera were employed in this work. The sera were inactivated at 56°C. for 40 minutes before use.

Throughout the extraction of streptococci with serum, the pH of a preparation is important, since if the pH is as high as 8.4 during the extraction process, only weak toxins are obtained. With sheep serum whose pH after inactivation is usually about 8.5, the pH was brought down to 8.2 with N HCl before use. Although the pH of both inactivated horse and rabbit sera may be as high as that of inactivated sheep serum, their pH's fall more rapidly than that of the sheep serum during extraction and so it was found unnecessary to adjust their reactions. To obtain the best results, the final pH of a preparation after extraction was found to be 8 to 8.1. When, after extraction, the pH of an extract was found to have fallen below pH 8, which often occurred when rabbit serum was used, it was raised to 8.1 with N NaOH. The reason for this last procedure is that although the toxins when freshly prepared with a final pH of 7.4 to 7.8 are exactly as toxic as those with a final pH of 8 to 8.1, they deteriorate more rapidly. Todd in his work on streptococcal serum hemolysin also noted that the hemolysin deteriorated faster at a pH of 7.4 than at pH 8 (8).

Preparation of Toxic Extracts.—Each tube of medium was inoculated through the vaseline seal with 0.1 cc. of a 1–50 dilution of streptococcus pleural exudate. After 13 hours growth, the tubes were chilled and centrifuged at high speed until the supernatants were clear. Then the supernatants were pipetted or poured off and the sedimented streptococci from each tube were taken up in 2 cc. of inactivated serum. The serum suspensions of streptococci were pooled, glass beads added, and they were shaken in a slow shaking machine for 1 hour. No growth of this strain of hemolytic streptococcus occurs in inactivated undiluted serum. After shaking, the material was chilled and centrifuged. Finally the clear supernatant was passed through a Chamberland-Pasteur filter L₂ for sterilization purposes. No poisonous properties were lost by such filtration. The recovered filtrate was the toxic extract used in this work.

The process of extraction of the same streptococci with untreated serum may be repeated twice if all 3 extractions are carried out on the same day. Under

these conditions, all 3 extracts are of approximately equal potency. More than 3 extractions have not been attempted.

Hemotoxin Tests.—Tests for hemotoxin were carried out in the usual way. Dilutions of the filtrate to be tested were made either with broth or with physiological salt solution. 1 unit of hemotoxin represented the smallest amount of filtrate which completely hemolyzed 2 cc. of 1 per cent well washed rabbit red cells in 1 hour at 37°C. in a water bath.

Leucocidin Tests.—The leucocyte suspensions for the leucocidin tests were obtained from rabbits following the intrapleural injections of aleuronat. 18 hours after injection, the animals were exsanguinated from the carotid artery, under ether anesthesia, and the pleural exudates procured from them were immediately centrifuged slowly for 3 minutes. Nine-tenths of the slightly clouded supernatant was discarded, leaving the leucocyte concentration approximately 10 times that of the original pleural exudate. Only those exudates showing no reddish color and containing very few if any red cells were used in the tests. The concentrated exudates were then titrated to determine the proper amount to use in the leucocidin tests. For this purpose, 0.1 cc. of 1–5,000 dilution of methylene blue was added in varying amounts to the leucocyte suspension in large precipitin tubes and the volume in each tube brought up to 0.5 cc. with saline solution. Vaseline seals were added to all the tubes and then they were put in a water bath at 37°C. The quantity of the leucocyte suspension used in the leucocidin tests was the amount that reduced the methylene blue completely in 20 minutes. The leucocyte suspension was diluted with broth so that 0.1 cc. contained the required amount.

The leucocidin tests were set up as follows: 0.1 cc. of leucocyte suspension was added to varying amounts of streptococcal serum extract and the volume of all tubes made up to 0.5 cc. with saline. All tubes were placed in the water bath at 37°C. for 1½ hours, during which time they were taken out and shaken at intervals of 10 minutes. Then 0.1 cc. of 1–5,000 dilution of methylene blue and a vaseline seal were added to each tube; and finally they were reincubated for 1 hour. 1 unit of leucocidin represented the minimum amount of a filtrate which completely prevented reduction of methylene blue by the leucocytes in 1 hour.

EXPERIMENTAL

Toxic Properties of Serum Extracts of Streptococci. Toxicity for Mice.—Mice were injected intravenously through a tail vein with the toxic filtrates. Generally 0.1 to 0.2 cc. of a filtrate brought about death within 24 hours. With larger amounts of a toxic filtrate, death occurred within 1 hour. The symptoms were dyspnea, weakness, and prostration. When the animals survived 1½ hours, deep red urine was passed. This appeared 50 minutes to 1½ hours after the inoculation.

At autopsy in those animals which died quickly, that is within 1 hour, nothing abnormal was seen macroscopically. In the majority of those which survived longer (2 to 8 hours) the bladder was large, tense, reddish black, and contained several cubic centimeters of very red urine. In the others, the bladder was small but almost always a slight amount of bloody urine was present. In animals which survived still longer (8 to 48 hours) generally all the organs and tissues were pale. Sometimes there was a slight amount of blood still present in the urine. The red color of the urines was shown to be due to the presence of hemoglobin. The pathology of mice dying of the serum extract toxin will be taken up in a later publication. M'Leod and M'Nee also noted hemoglobinuria and anemia in rabbits inoculated with large doses of streptococcal hemotoxic filtrates (4).

Normal inactivated serum (sheep, horse, or rabbit) had no toxic effect when injected intravenously into mice in 0.5 cc. amounts, nor had serum extracts of young cultures of other organisms (hemolytic *Staphylococcus aureus*, virulent Pneumococcus III, *B. typhosus*) produced by the same method as that used in the preparation of toxic extracts of streptococcus, any toxicity for mice. These latter preparations also contained no hemotoxin.

Experiment 1, Table I, gives one of our protocols on the effect of a toxic extract on mice.

This toxic extract was made with inactivated sheep serum. The pH of the filtrate was 8.1. It contained 200 hemotoxic units per cc.

The control serum was some of the same inactivated sheep serum as that used for the production of the toxic extract. Before use its pH was brought to 8.1 with N HCl and it was filtered through a Chamberland-Pasteur filter. Both the toxic extract filtrate and the control serum filtrate were sterile.

Hemotoxic Activity of Toxic Extracts.—All the serum extract filtrates were titrated for hemotoxin. Freshly prepared extracts which were toxic for mice in small amounts were always markedly hemolytic. These preparations contained between 150 and 330 hemotoxic units per cc. The question of the relationship between the lethal and the hemotoxic content of a filtrate will be taken up in a later part of this paper.

Leucocidic Activity of Toxic Extracts.—Relatively few of the toxic

extracts were titrated for leucocidin. However the results obtained in these were clear-cut. Usually a toxic filtrate contained 10 leucocidic units. An interesting phenomenon occurring in these tests was the clumping of the leucocytes in sticky masses in some of the tubes containing the toxic extracts. This necessitated frequent shaking of

TABLE I

Inoculation of Mice with a Serum Extract of Hemolytic Streptococci

Mouse No.	Weight	Material inoculated intravenously	Symptoms	Died or survived	Autopsy
	gm.				
1	16.5	Control serum 0.5 cc.	0	S	Killed with ether 3 days after inoculation. Autopsy negative
2	15.0	Control serum 0.4 cc.	0	S	Killed with ether 3 days after inoculation. Autopsy negative
3	14.5	Toxic filtrate 0.4 cc.	++++	D 8 min.	Negative
4	17.2	Toxic filtrate 0.4 cc.	++++	D 8 min.	Negative
5	17.2	Toxic filtrate 0.3 cc.	++++	D 40 min.	Subcutaneous tissues and peritoneum deep pink. Intestines congested. Urine negative
6	14.0	Toxic filtrate 0.3 cc.	++++	D 1½ hrs.	In ice box overnight. Intestines congested. Urine negative
7	17.0	Toxic filtrate 0.2 cc.	++++	D 5 min.	Negative
8	15.0	Toxic filtrate 0.2 cc.	+++	D 18 hrs.	Post mortem. Slight amount of red urine in bladder. Liver pale
9	17.7	Toxic filtrate 0.1 cc.	++	D 26 hrs.	Liver pale. Urine negative
10	15.0	Toxic filtrate 0.1 cc.	++	S	For 2 days very sick—then better. Killed with ether 3 days after inoculation. Liver pale brown. Few yellowish areas on surface. Kidneys dark brown

the tubes during the first incubation in order to bring the leucocytes in contact with the toxin. In the tubes containing strong undiluted toxin, the leucocytes were dissolved and the tubes cleared.

Control tubes containing undiluted or diluted filtered inactivated serum brought to pH 8.1 had no effect or only a very slight effect in

delaying the reduction of the methylene blue by the leucocytes. Sometimes there was a slight sedimentation of the leucocyte suspensions, which, however, could be resuspended easily by shaking. The question of the relationship of the leucocidin to the other toxic properties of the toxic extracts is not taken up in this paper.

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TABLE II

Titrations of a Serum Extract Filtrate for Leucocidin

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Toxin A.—Horse serum extract filtrate, pH 8.1.

Toxin B.—Toxin A, to each cubic centimeter of which was added 0.05 cc. of hemolyzed red cells (0.1 cc. packed red cells, 2 cc. distilled water, centrifuged, used clear supernatant). Toxin B was prepared because of Evans' (2) work showing that washed red cells increase the production of leucocidin in broth culture filtrates of hemolytic streptococci.

Tube No.	Toxin	Amount	Broth	Leucocytic suspension		Amount of reduction in				
						10 min.	20 min.	30 min.	1 hr.	1½ hrs.
		cc.	cc.	cc.						
1	A	0.4	—	0.1		0	0	0	0	0
2	A	0.3	0.1	0.1		0	0	0	0	0
3	A	0.2	0.2	0.1	1½ hrs. at 37°C. in	0	0	0	0	0
4	A	0.1	0.3	0.1	water bath—then added 0.1 cc. of	0	0	±	+	+
5	B	0.4	—	0.1	1–5,000 dilution of	0	0	0	0	0
6	B	0.3	0.1	0.1	methylene blue and	0	0	0	0	0
7	B	0.2	0.2	0.1	vaseline seals to each	0	0	0	0	0
8	B	0.1	0.3	0.1	tube and reincubated	0	0	±	+	+
9	Hemoglobin in broth	0.4	—	0.1		0	++	+++	+++	+++
10	—	—	0.4	0.1		++	+++	+++	+++	+++

± to +++ indicates amount of reduction of methylene blue.

+++ indicates complete reduction.

Experiment 2, Tables II and III, gives a leucocidin and a hemotoxin titration of a toxic extract filtrate.

After 10 minutes incubation (1st incubation) the contents of Tubes 1 and 5 had slightly cleared and suspensions in Tubes 2, 3, 6, and 7 had clumped. After 1 hour Tubes 1 and 5 were almost clear and the leucocytes in Tubes 2, 3, 4, and

6, 7, and 8 were clumped into sticky masses that were hard to break up by shaking. Tubes 9 and 10 showed only a slight sedimentation of the leucocyte suspension.

Smears were made from all the tubes after 1½ hours incubation (1st incubation) and stained with Jenner stain. In all the tubes excepting the controls (Tubes 9 and 10) the leucocytes had disintegrated or stained badly. The leucocytes appeared normal in Tubes 9 and 10.

In this experiment there seems to have been no difference in the amount of leucocidin in Toxins A and B; in other words, the presence of hemoglobin did not cause an increase of leucocidin. In another experiment, using a different extract, the addition of hemoglobin was also without effect.

TABLE III

Titration of Serum Extract Filtrates A and B for Hemotoxin

Toxin	30 min.					1 hr.				
	0.01	0.008	0.006	0.004	0.002	0.01	0.008	0.006	0.004	0.002
A	++++	++++	++++	++++	++	++++	++++	++++	++++	+++
B	++++	++++	++++	+++	++	++++	++++	++++	++++	+++

++ indicates hemolysis of approximately 1/2 to 3/4 of cells.

+++ indicates hemolysis of approximately 3/4 of cells to complete hemolysis.

++++ indicates complete hemolysis.

Biological Properties of the Streptococcus Toxic Filtrates

Stability and Preservation.—Heating a toxic extract to 56°C. for 30 minutes destroys virtually all the lethal and hemotoxic actions of a filtrate; and heating to a 56°C. for 2 hours almost completely destroys its leucocidic activity. The toxic properties of an extract of pH 8.1 usually remain stationary at ice box temperature for 24 hours, but if an extract is kept longer than this, it deteriorates rapidly, irrespective of whether or not it is covered with vaseline. Attempts were made to preserve the toxic activities of extracts by keeping them in jars of hydrogen or by reduction with 0.1 per cent $Na_2S_2O_4$ or by both these methods.[1] Our results indicate that the $Na_2S_2O_4$ did delay slightly the deterioration of the lethal and hemotoxic activities of a preparation but that the hydrogen had no effect in postponing the deterioration.

Immunology of Toxic Filtrates.—The question of the antigenicity of the toxic extracts was next considered. Numerous attempts to produce streptococcal antihemolysin have resulted in negative experi-

[1] We are indebted to Miss Ruth Pauli for setting up the hydrogen jars for us.

ments until the recent discovery of Todd (5) showing that the presence of serum in culture media for the preparation of streptolysin modifies the lysin, making it non-antigenic, whereas streptolysin prepared by growing streptococci in media without serum is an active antigen. From this work it was natural to expect that streptococcal serum extract filtrates also would not stimulate the production of anti-hemotoxin.

Our experiments along this line of work are only preliminary and therefore incomplete. We attempted to immunize 3 series of mice with serum extract toxins, using a toxin prepared with a different kind of serum for the test inoculation. These experiments were entirely negative; viz., the immunized mice showed no more resistance to the toxin than the controls. We also tested several different antistreptococcal sera for the presence of neutralizing antibodies for the lethal toxin and the hemotoxin of streptococcal serum extract. Again our results were negative.

These experiments, so far as they go, appear to indicate that the lethal and the hemotoxic principles of the extracts are not antigenic. We expect to extend the researches on the immunology of streptococcal extract filtrates in a later work.

What Substance or Substances in the Toxic Filtrates Cause Death of Mice?

A question of interest was: Is death of the mice due to the anemia produced by the hemotoxin *per se*, or to some condition resulting from the anemia it causes, or is death due to some other toxic principle or principles in the toxic filtrates?

In the first place, it was conclusively proved that the sickness and death of mice inoculated with toxic extracts, were not due to the toxic properties of the hemoglobin liberated during the hemolysis of the red cells. Large amounts of hemolyzed red cells (0.5 cc. of a 1–2 dilution of ether-hemolyzed packed rabbit red cells) were not toxic when injected intravenously into mice. Such an amount of hemoglobin caused hemoglobinuria similar to that obtained after inoculation of a toxic extract.

Secondly, it was demonstrated that extreme anemia is produced in mice inoculated with toxic extracts, by the red cell counts obtained in a series of 6 toxin-injected mice. Tables IV and V give the results of this experiment.

The toxin used in the experiments (Tables IV and V) was a comparatively weak sheep extract filtrate, both as to lethal and hemotoxic content, the latter being 150 units per cc.

The results of this experiment demonstrate that all the mice had severe anemia after the injection with the exception of Mouse 1, which became neither anemic

TABLE IV

Toxic Effect on Mice of Intravenous Injection of Streptococcus Serum Extract Filtrate

Mouse No.	Weight	Amount of toxin injected	Symptoms	Died or survived	Remarks
		cc.			
1	17.5	0.2	0	S	Never sick
2	19.0	0.3	++++	D 3 hrs.	
3	19.5	0.2	+++	S	For 3 days very sick. June 26, well
4	22.0	0.25	++++	D 4 hrs.	
5	21.5	0.25	+++	D 6 hrs.	
6	18.0	0.3	+++	S	For 3 days very sick. June 26, well

TABLE V

Anemia Produced in Mice Inoculated with Toxic Extracts (See Table IV)*
R. B. C. counts in thousands per cubic millimeter

Date	Time	No. 1	Time	No. 2	Time	No. 3	Time	No. 4	Time	No. 5	Time	No. 6
1933												
June 12		8,950		6,980		7,870		6,740		7,210		6,870
June 13		8,888		6,688		7,210		7,195		7,368		6,970
June 14		7,880		7,460		7,930		7,910		6,970		7,850
June 17		6,890		6,410		7,290		7,320		6,930		7,050
Inoculated at 11 a.m. June 22. (See Table IV.)												
June 22	3 p.m.	7,610	1 p.m.	1,860	1 p.m.	3,850	1 p.m.	2,170	3 p.m.	2,760	1 p.m.	2,610
June 23	10 a.m.	7,800		D 3 hrs.	10 a.m.	2,980		D 4 hrs.		D about 6 hrs.	10 a.m.	2,300
June 26	4 p.m.	6,800			4 p.m.	4,810					1 p.m.	3,870

* We are indebted to Miss Margaret Prest for making the R. B. C. counts in this experiment.

nor sick.   The most probable explanation of this discrepancy is that the inoculation was given subcutaneously instead of intravenously.

From these observations it appears that if the toxicity of the extracts for mice is due to the presence of hemotoxin in such extracts, death is brought about by the anemia itself or by some condition resulting from the anemia and not by the presence of hemoglobin *per se*.

But if death of the mice is caused by anemia, it would be logical to suppose that there would be a correlation between the hemotoxic and lethal properties of a preparation. Such, however, is not the case. In a series of 30 different streptococcal filtrates, titrated both for hemotoxic and lethal principles, 1 lethal unit of toxin contained anywhere from 11 to 100 units of hemotoxin. This series contained freshly prepared toxins which were usually strong in both lethal and hemotoxic properties, and weaker toxins, which had been deteriorated through standing or slight heating (37°C. for 1 hour or 56°C. for 5 minutes) and which were usually relatively stronger in lethal than in hemotoxic content per cubic centimeter. This last observation seems to indicate that the lethal principle is relatively more stable than the hemotoxic principle.

In connection herewith, it is interesting to note that the relationship between the lethal and hemotoxic content of a streptococcal serum extract preparation is in direct contrast to that obtained between the lethal and hemotoxic content of staphylococcal toxic filtrates. Here 1 lethal unit in a weak preparation contained usually twice as many hemotoxic units as in a strongly toxic preparation (6).

The preceding evidence on the lack of parallelism between the hemotoxic and lethal activities of streptococcal serum extracts appears to signify that there is some toxic substance other than the hemotoxin in such extracts which causes death; in other words, that the injected mice do not die solely of anemia or some condition resulting from the anemia. At present we have no experiments bearing on whether this other toxic substance is leucocidin.

Extraction of Hemolytic Streptococci with Diluted Serum and with Materials Other than Serum

Attempts were made to extract toxic substances from hemolytic streptococci with diluted serum and with various other materials.

Inactivated serum diluted 1–2 or 1–5 with broth is a fairly strong extracting agent for hemotoxin, the most potent preparation obtained containing 160 units per cc. Serum diluted 1–5 with distilled water, boiled for 10 minutes, and brought to isotonicity with NaCl also extracts hemotoxin from streptococci, in one experiment 80 units per cc. However, filtrates from the latter preparations (diluted serum and diluted boiled serum extracts) did not kill mice in 0.5 cc. amounts injected intravenously. Both egg white diluted 1–2 with broth, and milk heated to 56°C. for 40 minutes extracted about 60 units of hemotoxin per cc. from hemolytic streptococci. Broth was a weak extracting agent for hemotoxin (extracted 20 units per cc.) and dextrose 1 per cent, physiological saline, or gelatine 2 per cent in saline, extracted no hemotoxin from streptococci. These latter extracts were not tested intravenously in mice.

DISCUSSION

This work demonstrates that sterile filtrates of certain serum extracts of hemolytic streptococci are leucocidic and extremely hemolytic and cause death of mice when injected intravenously in comparatively small amounts. Whether the toxic properties of these extracts are due to one or to several substances has not as yet been conclusively proved.

It is interesting to note here how the origin of hemotoxin differs with various organisms. In the pneumococcus, hemotoxin is endocellular and is only liberated by the breaking up of the cells (7); in *Staphylococcus aureus* it appears not to be endocellular, nor is it on the surfaces of the organism (if our negative results with the serum extraction of the staphylococcus signifies this) but is given off during the growth in a suitable medium, perhaps either as a secretion product or as a derivative product of some substance in the medium; on the other hand, from this work, the hemotoxin of *Streptococcus hemolyticus* appears, in great part at least, to be easily extractable. It is possible that hemotoxin may be liberated in some way by the breaking up of the streptococci during the extraction process, but if there is a disintegration of the cocci, it must be slight, since in stained smears of the extracts after shaking, they appear to be intact.

CONCLUSIONS

1. A method is described whereby toxic substances may be extracted from hemolytic streptococci with inactivated serum.

2. Such extracts contain large amounts of hemotoxin and leucocidin.

3. Their intravenous injection into mice causes marked hemoglobinuria, anemia, and death.

4. There is evidence that this anemia is not the only cause of death of these animals.

5. Incomplete work seems to indicate that the hemotoxin and the lethal poisons are not antigenic.

6. Certain biological properties of the extract are described.

We are indebted to Miss Anne Gunther for technical assistance in the first part of this work.

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FURTHER OBSERVATIONS ON PATHOLOGIC SIMILARITIES BETWEEN EXPERIMENTAL SCURVY COMBINED WITH INFECTION, AND RHEUMATIC FEVER*

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PLATES 9 TO 12

In recent preliminary communications (1) experimental data were presented showing that degenerative and proliferative lesions may be produced in the heart valves of the guinea pig by subjecting the animal to the combined influence of scurvy and a localized infection with hemolytic streptococci. Attention was directed to the fundamental similarity of the lesions so produced to those of rheumatic fever. Proliferative reactions were observed in the myocardium and pericardium that lent more support to the analogy. In addition it was briefly pointed out that a more or less prolonged inadequacy of vitamin C in the diet produces functional impairment and anatomic changes in the joints of the experimental animals. When the insult of infection was added to this disability the functional impairment and anatomic changes were accentuated. The lesions in the joints were noted to be of a type consistent with those of rheumatic fever. No physiologic impairment or lesions developed in the joints of animals subjected to the same infection if maintained on a diet with adequate vitamin C supplement. This suggestive experimental evidence naturally led to an analysis of other data which might support the view that scurvy may bear a relation to rheumatic fever. It was pointed out that such a concept would appear to afford an explanation for the epidemiological peculiarities of rheumatic fever. Certain similarities in the symptomatology of latent scurvy and the prerheumatic or early

rheumatic state were noted. It was suggested that a condition of latent scurvy may provide the susceptible host and when the factor of infection is added to the scorbutic state, the symptoms and lesions of rheumatic fever develop. A detailed description of the lesions resembling those of rheumatic fever produced in the heart valves and heart muscle by combining infection and scurvy in the guinea pig has been reported (2). Other lesions notably those in joints produced by combined scurvy and infection likewise bear rather striking resemblance to those of rheumatic fever. It is the purpose of the present communication to consider these pathologic changes. Particular attention is directed to a peculiar degenerative lesion involving collagen which appears to be a fundamental alteration common to both rheumatic fever and scurvy.

Outline of Methods

An outline of the essential facts of method will be reviewed. The basal diet employed was a modification of one that has been used extensively in researches on experimental scurvy in the guinea pig.[1] It is complete in all food factors except vitamin C and, when supplemented with adequate amounts of orange juice, is sufficient for growth and maintenance. The infecting agent was usually a hemolytic streptococcus derived from the suppurative cervical lymphadenitis which occurs spontaneously in the guinea pig. This organism is a favorable one because it is adapted to the guinea pig and will almost uniformly produce a localized subacute infection of the lymph nodes draining the site of inoculation. It rarely causes spontaneous death and the infection remains localized in most instances. Infection was transferred by intracutaneous inoculation of 0.05 to 0.1 cc. of a 24 hour culture of the organism grown on Avery's glucose veal broth. The inoculations were made into the skin at the inner aspect of the thigh. The experiments performed included a study of the pathological effects of acute, subacute, and chronic scurvy alone, of similar degrees of scurvy combined with infection, and,

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[1] The basal diet used has the following composition:

	per cent
Baked skimmed mild powder (baked at 110–120° for 2 hrs.)	30
Ground rolled oats and bran—equal parts by volume	56
Butter fat	10
Dried yeast (Fleischmann's yeast for animals and poultry)	1.5
Cod liver oil (standardized)	1.0
Sodium chloride	1.0
Ferrous lactate	0.5

as controls, infected and non-infected animals maintained on the same basal diet adequately supplemented with orange juice.[2]

Lesions in the Joints

Data available at the present time with respect to changes in joints of guinea pigs maintained under the influence of combined scurvy and infection are perhaps not adequate for final conclusions, but the relatively few observations are so highly suggestive that they are worthy of record.

Joint pain and swelling are well known to be a part of the symptomatology of scurvy. Joint manifestations, chiefly stiffness and partial fixation in the knees, were noted in early experiments in the animals subjected to subacute or chronic scurvy both with and without infection, although systematic observations were not made. In a recent experiment performed particular attention was given to the joint manifestations and pathology. In this series there were seven animals maintained on the basal diet adequately supplemented with orange juice and infected with *Streptococcus hemolyticus.* Four animals were maintained in a condition of chronic scurvy on a diet severely but not wholly deficient in vitamin C. Nine animals were maintained on the same vitamin C-deficient diet and subjected to infection with *Streptococcus hemolyticus.*[3] The animals were maintained on the

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[2] 4 to 6 cc. daily has been considered an adequate supplement for animals weighing from 300 to 450 gm. The acutely scorbutic animals were deprived entirely of vitamin C. Animals with subacute and chronic scurvy received small but inadequate vitamin C supplements to the diet. Those with chronic scurvy were maintained for longer periods of time than the subacute groups.

[3] The exact dietary régime and experimental procedure in the chronic scurvy animals in this series can be seen from the following protocol. The animal is one in which infection was superimposed upon the scurvy.

Animal 152. Weight 408 gm. Placed on basal diet beginning Jan. 19, 1933. Maintained for 67 days with a total of 24 cc. of orange juice supplement (6 cc. first 13 days, none the next 19 days, an average of 1 cc. daily for 11 days, none the next 7 days, and total of 7 cc. during the last 25 days). Inoculated 0.1 cc. broth culture *beta* streptococcus into skin of left thigh on Feb. 10, 1933. Feb. 19, 1933, infection maximal with large mass in left groin 2 x 1.3 cm. On this day noted swelling and fixation of right knee and fixation of left knee. Infection persistent, slow regression. Animal chloroformed Mar. 27, 1933. Weight 272 gm. Pathological notes: Small lymph nodes in both groins. Patchy atelectasis of lungs. Costochondral junctures swollen. Single abscess in spleen. Bladder hemorrhagic. Brownish discoloration (old hemorrhage) about right knee. No evidence of recent hemorrhage. Questionable shortening of mitral valve. Subcutaneous

dietary régime for 22 days before infection. Infection was transferred by inoculation into the skin of the left thigh of 0.1 cc. of a 24 hour broth culture of *Streptococcus hemolyticus* (source—spontaneous cervical adenitis of the guinea pig). Observations were made on the joints in the entire series 9 days after the transfer of infection.

Of the seven animals subjected to infection but maintained with an adequate vitamin C supplement to the diet no involvement of the joints was observable. (Two animals showed partial fixation of the left knee not due to intrinsic joint pathology but to tumefaction of the regionally infected lymph nodes.)

Of the four animals of the chronic scurvy group, non-infected, two showed stiffness and partial fixation of the knees (see Fig. 1). Of the eight surviving animals subjected to chronic scurvy and infection all showed some fixation of the left knee (on the side of inoculation). Three, in addition, showed well defined swelling of both knees as illustrated in Fig. 2.

In one instance the swelling was due to a suppurative arthritis; in the others not.

It is believed that these observations, although including but a relatively small number of animals, show not only a definite arthropathy in scorbutic animals but a tendency for a more manifest arthropathy in the scorbutic animals subjected to infection. Further experiments are in progress to extend these observations. It would appear probable that other infecting agents than the streptococcus used might produce similar effects.[4]

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nodule over bony prominence of knee. Microscopic examination shows definite interstitial proliferation in mitral valve. The subcutaneous nodule shows bands of fibrinoid degeneration and endothelial proliferation resembling the lesion of rheumatic fever. Joints show fibrinoid degeneration in synovia and periarticular tissues. Similar degeneration in connective tissues about costochondral junctures. Much hemosiderin in the spleen. Hemosiderin and erythrophagocytosis in cervical lymph nodes.

[4] A series of animals under observation at the present time would appear of interest in this connection. In the group there are twelve animals that have been maintained on a chronic scurvy-producing diet for 33 days. The animals have received 1 cc. of orange juice three times weekly. Six of the animals were subjected to infection by inoculation into the skin of the neck 27 days after the onset. Observation of the entire group was made on the 33rd day. Of the chronic scurvy group, without infection, all showed some stiffness and slight thickening of one or both knees. One animal only showed a mild swelling of one knee. Of the chronic scurvy group that were infected four showed in addition to the stiffness and thickening a moderate swelling of one or both knees, and one animal exhibited a mild swelling. Only one animal showed the simple stiffness and thickening of the non-infected group. Judged by the local reaction at the site of inoculation the infection

Histologic observations include a somewhat larger series of animals. One or more joints were examined microscopically in 29 animals as follows: Acute scurvy 3, subacute or chronic scurvy 4, acute scurvy plus infection 1, chronic scurvy plus infection 7, infection followed by subacute scurvy 4, control (adequate) dietary without infection 3, and control (adequate) dietary with infection 7.

Histologic observations were made on the joint cavity, the synovia, and the capsular and periarticular tissues. One observation of considerable significance is that the animals infected but maintained on a diet with adequate vitamin C supplement show no physiological impairment or gross or microscopic lesions in the joints. Fig. 3 shows a normal knee joint in such an animal. Note the clear joint space and simple synovial lining.

In acute scurvy the changes observed in the joints were minimal. A little hyaline fibrinous material was seen in the joint space adherent to the synovia which showed no or only very slight proliferation. A mild degree of hemorrhage was seen in the joint capsule in one instance. The single animal subjected to acute scurvy and infection showed essentially the same picture.

In chronic scurvy more definite and severe lesions were seen, in agreement with the greater functional impairment observed. Considerable amounts of a hyaline fibrinous material were seen in the joint spaces usually adherent to or replacing the synovia. In a few instances a slight proliferation of the synovial tissue was observed. In the subsynovial, capsular, and periarticular tissues, streaks and masses of a peculiar, brilliant eosinophilic hyaline material were frequently encountered. Areas of hemorrhage were also present.

There did not appear to be any fundamental difference in the character of the joint lesions in the animals subjected to chronic scurvy alone and those subjected to both scurvy and infection except that the lesions in the latter group were in general more pronounced.

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in this group of animals was quite mild. Because of the unusually mild local reaction, cultures were made from the neck glands and tissues of several animals. An organism which gave a greyish white semitranslucent colony and produced hemolysis on blood agar was isolated in most instances. This organism was identified as a staphylococcus by fermentation reactions. The usual hemolytic streptococcus was not present.

Fibrinous hyaline material was invariably present in the joint spaces and the synovial proliferative reactions were more striking than in animals subjected to scurvy alone. Foci of hyaline eosinophilic material were regularly seen in the subsynovial, capsular, or periarticular tissues. Fig. 4 shows a characteristic picture of the knee joint in an animal subjected to the combined insult of chronic scurvy and infection. Dark tongue-like masses, attached to and extending from the synovia may be seen in the joint recesses. In sections stained with hematoxylin and eosin this dark material takes a brilliant eosinophilic stain resembling fibrin. The regional synovial lining is mildly hyperplastic and there is some fibroblastic extension into the hyaline material. (Contrast with normal knee joint of animal subjected to infection but maintained on an adequate diet shown in Fig. 3.) Fig. 5 illustrates a portion of the knee joint beneath the patellar tendon in another animal subjected to subacute scurvy and infection. The hyaline material extending into the joint space, the synovial proliferation, and the hyperplastic connective tissue reaction above and below the patella tendon are well shown. A portion of the same lesion is illustrated in greater detail in Fig. 12, *b*. Here the eosinophilic fibrinoid material may be seen extending into the subsynovial tissue. Fig. 7 shows a tongue-like projection from a recess in the knee joint composed of an intimately intermingled hyaline fibrinoid material and proliferated synovial cells. Figs. 6 and 8 illustrate areas of hyaline (fibrinoid) degeneration in the periarticular tissues.[5]

Similarity of the Joint Pathology to That of Rheumatic Fever

Detailed observations on the joints in acute rheumatic fever are not abundant. Fahr (3) described in acute rheumatic fever a proliferation of the synovia with the lumen side bordered by a fibrinoid necrotic material and a rheumatic nodule in a tendon in the region of the knee joint showing a fibrinous hyaline material lying between the fixed elements. The full significance of the peculiar pathologic changes present in the joints of the experimental animals subjected to chronic scurvy and infection was not appreciated until the careful observations of Klinge (4, 5) and Klinge and Grzimek (6) were studied. Klinge (4) in a detailed account of the pathologic findings in a case of rheumatic fever recorded changes in the joints typified by hyperplasia of the synovial epithelium which was layered and interrupted by nodes and streaks of fibrinoid necrosis. The connective tissue was edematous and stippled with leukocytes. The subsynovial and capsular connective tissues contained foci of fibrinoid degeneration. In subsequent studies Klinge and Grzimek (6) and Klinge (5) extended and amplified the observations on joints in rheumatic fever. The lesions in rheumatic arthritis were characterized by fibrinoid degeneration and granulomatous reactions in the capsular and periarticular tissues, some hyperplasia of the synovia with fibrinoid degeneration

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[5] All illustrations of joints except Fig. 3 are from animals subjected to subacute or chronic scurvy combined with infection.

in the synovial and subsynovial tissues and the presence of hyaline fibrinous material free in the joint space. The intimate intermingling of the fibrinoid material with the synovia and extension into the subsynovial tissue were considered characteristic of rheumatic arthritis. We have been greatly impressed by the essential similarity of the experimental lesions and the lesions of rheumatic arthritis described and illustrated by Klinge.[6]

Klinge and Grzimek (6) hold that although acute or subacute rheumatic fever and chronic infectious or rheumatoid arthritis may usually be differentiated, both disease pictures are so intimately related in joint and general pathology that a rheumatic basis may be assigned to both.

Subcutaneous Nodules

Subcutaneous nodules were not looked for in routine postmortem examination of animals, yet one was encountered overlying the head of the fibula in an animal subjected to chronic scurvy and infection.

The nodule measured about 2 mm. in diameter. Histologically it is characterized by an intermingling of strands and masses of a brilliant eosinophilic substance, fibroblasts, and delicate endothelial channels. Fig. 9 shows the general architecture of the lesion. The drawing, Fig. 12, c, shows the peculiar hyaline degeneration accompanying the proliferative reaction.

Swift (7) in describing a subcutaneous nodule in rheumatic fever states, "In close apposition to areas of cellular proliferation there is tissue destruction varying in size from submiliary areas to long strands of hyaline necrosis affecting connective tissue fibres; combined with the necrosis are deposits of fibrin." Klinge (5) considers the subcutaneous rheumatic nodules essentially similar to the smaller areas of fibrinoid degeneration and granulomatous reactions encountered in the periarticular tissues; the subcutaneous nodules are only exaggerated lesions of the same sort. As has been previously noted, such lesions are characteristic findings in the periarticular tissues of the experimental animals and have been noted with considerable frequency in the tissues surrounding the costochondral junctures (Fig. 10).

Miscellaneous Pathological Lesions

Certain miscellaneous lesions which appear to have a common occurrence in rheumatic fever and in scurvy combined with infection, will be briefly mentioned.

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[6] The reader is referred to this author's illustration of the joint lesions of rheumatic fever in the following journals: *Virchows Arch. path. Anat.*, 1930, 278, 453, 459; 1932, 284, 655; 286, 363, 372.

Miscellaneous lesions commonly observed in rheumatic fever are skeletal muscular degeneration, foci of necrosis in the liver, hyaline degeneration of the follicle reticulum in the spleen, erythrophagocytosis in the lymph nodes, and focal accumulations of lymphocytes in the cortex of the kidney (5).

Comparable lesions were observed with considerable frequency in the experimental animals. Degeneration of the skeletal muscle is so frequent in the scorbutic animal as to be considered almost characteristic. This has been clearly shown by the work of Höjer (8), Meyer and McCormick (9), Dalldorf (10), and others. In our own animals degenerative changes in the skeletal muscle particularly in the intercostal muscles has been repeatedly observed in chronic scurvy and chronic scurvy combined with infection. Such a lesion, showing also a focus of fibrinoid degeneration and hyperplastic connective tissue is illustrated in Fig. 10. Focal necrosis in the liver has been another not infrequent observation in animals subjected to scurvy or to scurvy and infection. Hyaline thickening of the reticulum at the periphery of the lymph follicles of the spleen was commonly seen in the experimental animals subjected to scurvy and infection, particularly in a group which were maintained, after experimental infection (*beta* streptococcus), for a period of about 40 days on a diet severely deficient in vitamin C. Erythrophagocytosis in the lymph nodes was noted to be a characteristic finding in the animals subjected to combined scurvy and infection. Fig. 11 illustrates such a node. Although focal collections of lymphocytes are not uncommonly present in the cortex of the kidney of normal guinea pigs, in early experiments in which animals were maintained on a diet entirely without vitamin C and subjected to infection, we were impressed by the size and frequency of such reactions.

It would appear to be probably more than coincidence that this array of miscellaneous pathologic findings should have a common occurrence both in rheumatic fever and in scurvy or, more particularly, in scurvy combined with infection.

The Problem of Hemorrhage

Hemorrhage plays such a prominent part in outspoken scurvy that it is usually considered as a hemorrhagic disease. In the experimental animals studied, hemorrhagic manifestations were frequently noted. However, in the more chronic states although hemorrhages were usually present, the degree of hemorrhage was frequently not striking. The bladder and adrenal, and to a lesser extent, the periarticular tissues were frequent sites of gross or microscopic bleeding.

Hemorrhagic manifestations are not uncommon in rheumatic fever. Purpuric skin lesions have been recorded, and bleeding from the nose is of relatively frequent occurrence. Poynton and Paine (11) have described blood-stained synovial fluid

and a swollen hyperemic synovial membrane in acute rheumatic arthritis. The recent study of Coburn (12) would indicate that hemorrhage is characteristic of the acute phases of rheumatic fever. He describes nine cases examined early after the onset of an acute attack of rheumatic fever and notes the frequent occurrence of hemorrhages. He states, "In all the patients dying during marked activity of the rheumatic process, hemorrhagic lesions were widespread."

In cases of sepsis, hemorrhagic manifestations are of course common, but as pointed out by Coburn (12) the hemorrhages of rheumatic fever cannot be assigned to sepsis. A scorbutic factor in rheumatic fever would appear to offer a satisfactory explanation for the hemorrhagic manifestations.

Similarity of the Fundamental Pathology in Scurvy and Acute Rheumatic Fever

As has been previously indicated (2) basic lesions of scurvy and rheumatic fever have certain fundamental similarities.

The work of Aschoff and Koch (13), Höjer (8), and Wolbach and Howe (14) has demonstrated that the scorbutic animal is unable to form normal intercellular substances, which, of course, includes collagen. Höjer has emphasized the occurrence of degenerated or imperfectly formed collagen even in early and relatively mild degrees of scurvy. He considers that there is a stage in which the scorbutic animal, though unable to form a normal collagen, can elaborate an imperfect cement substance. The fibrinoid swelling of collagen which Klinge (4) holds to be the basic lesion in rheumatic fever would appear to represent a related degeneration or imperfect formation of collagen. Klinge has emphasized the widespread occurrence of this lesion in rheumatic fever. He considers it to be the initial and characteristic lesion of rheumatic fever occurring particularly in the heart valves, heart muscle, articular and periarticular tissues. The Aschoff reaction is a peculiar cellular response to this degenerative substance. We have noted (2) the occurrence of a degenerative change of this type in the heart valves. In one instance areas of similar degeneration were seen in the epicardial tissue adjacent to the heart muscle. This lesion is shown in Fig. 12, a. Lesions which are apparently identical with Klinge's fibrinoid degeneration appear to be the most characteristic changes occurring in the articular and periarticular tissues of animals subjected to chronic scurvy and more particularly those in which infection is combined with such a scurvy.

DISCUSSION

Experimental Attempts to Reproduce Rheumatic Fever.—Attempts to reproduce experimentally the lesions of rheumatic fever have proved disappointing. Gross, Loewe, and Eliasoph (15) have recently reviewed this subject and attempted themselves to reproduce the disease by injecting different strains of streptococci under a variety of

experimental conditions in a number of different species of animals. They concluded that not only were they unable to reproduce the disease but that the same could be said of the attempts of previous investigators. In general, most of the reported experiments involved intravenous inoculations with large doses of streptococci isolated in one way or another from cases of rheumatic fever. Lesions when produced experimentally in the heart valves corresponded more closely to those of bacterial endocarditis than to the unique lesions of rheumatic endocarditis. The joint pathology, The Aschoff reaction, or the subcutaneous nodules were not adequately reproduced. Klinge (16) and Vaubel (17) have more recently induced experimentally, pathologic changes bearing certain resemblance to those of rheumatic fever by repeated inoculations with foreign proteins.

The lesions produced by subjecting the guinea pig to the combined insult of scurvy and infection are considered by us to be fundamentally similar in character and distribution to those of rheumatic fever. This naturally suggests that some such relationship holds in man. Obviously, outspoken scurvy is not present in rheumatic fever. It would appear more probable that a subclinical or latent scurvy may form a background in which the added insult of infection precipitates rheumatic fever.[7] Although other factors would not appear necessary, such a concept does not deny the possible influence of intrinsic factors as hypersensitivity, or extrinsic environmental influences as dampness and cold.

The strongly suggestive experimental data naturally led to an analysis of the epidemiology of rheumatic fever to determine if it contained any evidence pointing toward a relationship between scurvy and rheumatic fever.

The epidemiology of rheumatic fever has long presented a challenge to the medical profession. Certain peculiarities of the disease, notably the social incidence, the seasonal incidence, and the geographical distribution, have awaited a satisfactory explanation. The studies of Swift (18), Paul (19), Poynton and Schlesinger (20), and Coburn (21) afford excellent sources of epidemiologic data on rheumatic fever.

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[7] Because of the close relationship known to exist between rheumatic fever and chronic infectious or rheumatoid arthritis it is believed that scurvy may play a rôle in this condition.

Influence of Malnutrition.—Numerous observers have emphasized the malnourished state of children suffering from, or that are prone to develop rheumatic fever. Vining (22) emphasizes the debilitated state of the prerheumatic child. This author suggested that vitamin B undernutrition might play a rôle in the development of rheumatic fever. Campbell and Warner (23) state, "We agree with Vining who maintains that it is the debilitated child who develops rheumatism." Swift observes that most rheumatic children appear undernourished.

Social and Urban Incidence.—It is a well recognized fact that rheumatic fever is predominantly a disease of the poor. Campbell and Warner (23) state that rheumatic disease is the most crippling affection of the children of the poor. Swift (24) notes that statistics show obvious rheumatic fever is fifteen to twenty times more frequent in the laboring classes than in those forming the bulk of private practice. The greatest incidence is among the poor of elementary school age living in the cities. One need only consider the notoriously ill advised diet of the poorer children of metropolitan areas such as New York and London to realize that malnutrition and probably latent scurvy might occur under such circumstances.

The Existence of Latent Scurvy.—The problem of the existence and incidence of latent scurvy has not yet received the attention it deserves. There are a number of reports of groups of cases of mild scurvy in the literature which will be cited subsequently. More recently there have been fundamental researches directed at the problem. Göthlin (25) using the reduced capillary strength as an index of latent scurvy found evidence of vitamin C undernourishment in about 18 per cent of the school children (between 11 and 14 years) in the province of Uppland (Sweden) during the months of April and May. In a subsequent study in the district of Norrbotten, north of the arctic circle, Falk, Gedda, and Göthlin (26) revealed a similar incidence of latent scurvy in presumably healthy children. Dalldorf (27), also using capillary resistance tests as a criterion, considers that mild degrees of vitamin C deficiency may constitute a problem of considerable public health importance. In a study of a group of children from poor homes in the State of New York he found an incidence of subclinical scurvy ranging from 35 to 66 per cent as estimated from capillary resistance derminations.

Such studies we consider of fundamental importance. The findings lend much support to the concept that scurvy may bear a relationship to rheumatic fever.

Age Incidence.—The age of onset of acute rheumatic fever is in the vast majority of cases under 15 years, as pointed out by Wilson, Lingg, and Croxford (28). Poynton (29) placed the average age of onset at 7 years and Coombs (30) at 10 years. This is a period of active growth in which the nutritional requirements are great. It has been recently shown (26) that children of elementary school age (5 to 14 years) require approximately two times more vitamin C intake per kilo for the prevention of latent scurvy than do adults. It would appear then that this would probably be the age of greatest incidence of subclinical or latent scurvy.

Seasonal Incidence.—Suggestive evidence of a probable relation of latent scurvy and rheumatic fever is present in the concurrent seasonal incidence of the two

conditions. All authors are agreed that the greatest incidence of rheumatic fever in this country is in the late winter and early spring. Campbell and Warner (23) point out that the maximum number of cases occur in England between November and April. This is the season following a period of diminished availability of fresh fruits and vegetables in which the greatest incidence of scurvy, either latent or manifest, would be expected to occur. This would be particularly true for poor people living in the cities. Falk, Gedda, and Göthlin (26) have pointed out that any reduction of the capillary strength (evidence of latent scurvy) is more likely to occur in the spring (April). In this connection it is of interest to note that Hart, Steenbock, and Ellis (31) have shown that the vitamin C content of winter milk (due to dry feed) was much inferior to summer milk. Dutcher *et al.* (32) found that 20 cc. of summer milk was superior in antiscorbutic properties to 60 cc. of winter milk. Considering the importance of milk in the dietary of children the finding would appear to be of considerable significance.

The Geographic Distribution.—The geographical distribution of rheumatic fever presents a fascinating problem. It has been maintained by Clarke (33) that rheumatic fever is non-existent in the true tropics. Seegal and Seegal (34) in a study of the geographic distribution of rheumatic fever in Canada and the United States showed that its incidence diminished as the tropics are approached. Clarke looked in the true tropics for rheumatic fever and did not find one case in more than 30 years. In contacts with about 150,000 patients he did not encounter a single case of rheumatic fever, mitral stenosis, or chorea. He quotes Sir Leonard Rogers (35) who writes that of 4,800 postmortem examinations in Calcutta only one case of rheumatic carditis was found in 37 years. Of India Sir Leonard Rogers (36) further states, "Heart diseases were half as common as in London and rheumatic endocarditis was quite absent, although streptococcal infections were otherwise as frequent." Indeed Clarke makes a strong case for the non-existence of rheumatic fever in the tropics. Whether or not one agrees with this extreme view, all are agreed that rheumatic fever is extremely rare in the tropics. Scurvy is rare in the tropics except under unsatisfactory conditions as those that sometimes prevail in labor camps or military campaigns. Rogers and Megaw (37) note that most of the dietaries of the tropics are quite well supplied with antiscorbutic substances except under such unusual circumstances. Indeed, if one analyzes the dietary of the various sections of the tropics one finds evidence that would render the occurrence of scurvy under ordinary circumstances extremely improbable. In the Dutch East Indies, the Malay Peninsula, and Central and South America the large amounts of fruits consumed probably prevent scurvy. In China and India it is usual to consume considerable amounts of green leafy vegetables including many of the cabbage variety known to be rich sources of vitamin C. The vegetables are, as a rule, subjected to very short cooking. This would preserve their antiscorbutic potency. Moderate amounts of milk products are also consumed. In the dry tropics, including North Africa, Southern Europe, Syria, Turkey, Arabia, and Central India, dairy foods form the dietary staple. They are consumed in

large amounts, are not pasteurized, and the producing animals graze on pasture lands.[8] Natural dairy foods in sufficient amounts are effective antiscorbutics. Recently Van Breeman (38) has reported the remarkable fact that the number of cases of rheumatic fever both in children and adults is comparatively small in Holland. Also cases of the closely allied rheumatoid arthritis are comparatively rare. It is interesting to speculate upon what influence dietary habits might have upon this. The importance of truck gardening and the dairy industry would suggest that the diet in this country would be more than usually adequate in vitamin C-containing foods.

Symptomatological Similarities of Latent Scurvy and Prerheumatic or Early Rheumatic State.—Symptoms of latent scurvy listed by Ohnell (39) are fatigue, mental depression, a feeling of oppression, dry skin, and perhaps anemia. Meulengracht (40) adds to the list irritability, palpitation, and loss of appetite. Friderichsen (41) describes a prescorbutic myopathy occurring in children which may simulate subacute rheumatism. Vining (22) in describing the prerheumatic child emphasizes the debility, diminished weight, loss of appetite, nervousness, pain in the limbs, headaches, and gastrointestinal disturbances. Coombs (42) describes the earliest symptoms of rheumatic fever as constitutional and characterized by loss of appetite, loss of weight, a vague seediness, and increasing pallor. Lewis (43) states that the children feel poorly, exhibit a loss of appetite, fatiguability, pallor, loss of weight, and frequently complain of pains in the limbs and chest. Miller's (44) descriptions are in agreement. We find then, general undernutrition, fatigue, loss of appetite, loss of weight, myopathy, nervousness, and anemia as symptoms common to both latent scurvy and the prerheumatic or early rheumatic state. The most accurate single index for the recognition of latent scurvy appears to be an estimation of the capillary resistance. A diminished resistance is characteristic of latent scurvy. A diminished resistance has been recorded by Stephan (45) and by Wiemer (46) in acute polyarthritis. This Wiemer considered to be due to the damaging effect of a toxin on the endothelium. A basic condition of latent scurvy in the rheumatic state would appear to offer a more satisfactory explanation.

Rôle of Infection in Rheumatic Fever.—There can be no reasonable doubt that infection plays an important rôle in rheumatic fever. The frequent occurrence of tonsillitis, sinusitis, and other upper respiratory infections preceding the onset of rheumatic fever is well known. Streptococci have been implicated by a great number of observers. Reference may be made to the Pickett-Thomson Research Laboratory report (47) for a historical survey of the bacteriological researches in rheumatic fever. Summaries of the bacteriological observations on rheumatic fever are also available in the reviews of Swift (18), Poynton and Schlesinger (20),

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[8] We are indebted to Dr. A. C. Reed, Professor of Tropical Medicine, University of California Medical School, and Dr. Nina Simmonds Estill for data regarding dietary habits in the tropics.

and Coburn (21). Briefly it may be said that a variety of organisms, chiefly streptococci, have been found associated with the disease. The streptococci found have, however, been of different sorts. The *Diplococcus rheumaticus* of Poynton and Paine (11) was probably a non-hemolytic streptococcus. Clawson (48) described strains of *Streptococcus viridans* isolated from twenty cases of rheumatic fever, rheumatic endocarditis, or chorea; thirteen of which were isolated from the blood. Cecil, Nicholls, and Stainsby (49) and others have reported isolation of *Streptococcus viridans* in a high percentage of blood cultures in rheumatic fever by a prolonged incubation technique. The organisms were, however, of different immunological types. Independent of the question of their etiological rôle, the difficulty encountered in growing the organisms would indicate that they are present in the blood in very small numbers. The investigations of Birkhaug (50) and Small (51) would implicate a non-methemoglobin-forming streptococcus. More recently, Swift (18) and Coburn (21) have brought into prominence the possible relation of acute upper respiratory infections caused by hemolytic streptococci to the development of rheumatic fever. All of the evidence points to a factor of infection in the genesis of rheumatic fever. The bacteriological findings, though implicating streptococci in general, show a lack of uniformity in types or strains. If, as the experimental data would appear to indicate, the scorbutic state is an important factor in the genesis of the lesions, a type- or strain-specific infection would seem unnecessary. Coburn stresses the variety of consequences resulting from implantation of the hemolytic streptococcus in the pharynx. Only in a small minority is local infection followed by the rheumatic state. The implication is clear, as others have noted, that a factor other than infection must play a dominant rôle. It is the concept of the authors supported by experimental, epidemiological, and clinical data that this other factor is a state of vitamin C malnutrition or latent scurvy. It would appear that two factors, one of infection and the other a state of latent scurvy are necessary for the development of rheumatic fever. If one thinks of the disease as a result of the two factors it can readily be seen that a mild scurvy with a severe infection or a more severe scurvy with a mild infection might produce a similar picture. The resulting disease would be the multiple effect of the two factors. An explanation would appear to be afforded for the known wide variability in severity. The definite diminution in the severity of rheumatic fever observed in the past 20 years could be explained on the basis of the known improvement in dietary habits that has come with our increased knowledge of nutrition. If the concept presented is correct, control would depend upon maintaining a high vitamin C nutritional status, and diminishing exposure to infection or eradicating established infections.[9]

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[9] It is interesting to consider that the amelioration of the disease that has been observed in cases transported to Porto Rico (21) might be due to a combination of the two beneficial effects.

SUMMARY

In the guinea pig, chronic scurvy with superimposed infection (*beta* streptococcus) and to a lesser extent chronic scurvy alone, produces an arthropathy with striking pathologic similarities to that of rheumatic fever and the closely allied condition of rheumatoid arthritis. Considerable significance is attached to the widespread occurrence in the experimental animal subjected to scurvy and infection, and to a lesser extent in scurvy alone, of lesions similar to if not identical with the fibrinoid degeneration which has been considered the fundamental lesion of rheumatic fever. A subcutaneous nodule essentially similar to the subcutaneous nodules of rheumatic fever was observed in one experimental animal.

Attention is called to a group of general pathologic changes frequently observed in rheumatic fever which were also found in the experimental animals subjected to scurvy and infection. These include degenerative changes in skeletal muscle, focal necrosis in the liver, fibrosis of the Malpighian bodies in the spleen, erythrophagocytosis in the lymph nodes, and focal lymphocytic accumulations in the kidneys.

The problem of hemorrhage is considered. It is suggested that a scorbutic state may be the basis of the hemorrhagic manifestations common to the acute phases of rheumatic fever.

The unsatisfactory nature of previous experimental attempts to reproduce the pathology of rheumatic fever is noted. The lesions produced by subjecting the guinea pig to the combined influence of scurvy and infection are considered to be fundamentally similar in character and distribution to those of rheumatic fever. The pathologic observations recorded in this and a previous publication (2) are believed to offer evidence that the disease known as rheumatic fever may be the result of the combined influence of scurvy and infection. It is suggested that a subclinical degree of scurvy may constitute the rheumatic tendency in which the added factor of infection causes the development of rheumatic fever or possibly the closely allied condition of rheumatoid arthritis.

Epidemiological and clinical considerations appear to afford supportive evidence to this concept.

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EXPLANATION OF PLATES

All of the sections were stained with hematoxylin and eosin.

PLATE 9

FIG. 1. Stiffness and partial fixation of knees due to chronic scurvy. Animal on left subjected to chronic scurvy. Animal on right, control. (Basal diet adequately supplemented with orange juice.)

FIG. 2. Fixation and swelling of knees due to chronic scurvy with superimposed infection. Animal on left, chronic scurvy plus infection. Animal on right, infection only. (Same infection but diet adequately supplemented with orange juice.) Knees normal.

FIG. 3. Knee joint. Infection only. Normal joint; note clear joint space and simple synovial lining. ×16.

PLATE 10

FIG. 4. Right knee, chronic scurvy plus infection. Note tongue-like projections of dark hyaline material extending from joint recesses. ×13.

Fig. 5. Joint recess beneath the patellar tendon. Subacute scurvy plus infection. Note the dark fibrin-like material extending into the joint cavity, the proliferation of the synovia, and the connective tissue reaction above and below the patellar tendon. ×72. (See also Fig. 12, b.)

Fig. 6. Periarticular tissue, right knee. Chronic scurvy plus infection. Note dark streaks of fibrinoid degeneration. ×195.

Fig. 7. Joint recess, right knee. Chronic scurvy plus infection. Intimate intermingling of dark hyaline, fibrinoid material and proliferating synovial and connective tissue cells. ×195.

PLATE 11

Fig. 8. Periarticular tissue, right knee. Chronic scurvy plus infection. Fibrinoid degeneration. ×195.

Fig. 9. Subcutaneous nodule. Chronic scurvy plus infection. Dark streaks of fibrin-like material are intermingled with proliferating fibroblasts and capillary endothelial cells. ×44.

Fig. 10. Intercostal muscle near costochondral juncture. Subacute scurvy plus infection. Muscle degeneration, connective tissue overgrowth, and focus of fibrinoid degeneration (on the right just above the middle of the photograph). ×195.

Fig. 11. Erythrophagocytosis in the sinus endothelial cells of a cervical lymph node. Chronic scurvy plus infection. ×195.
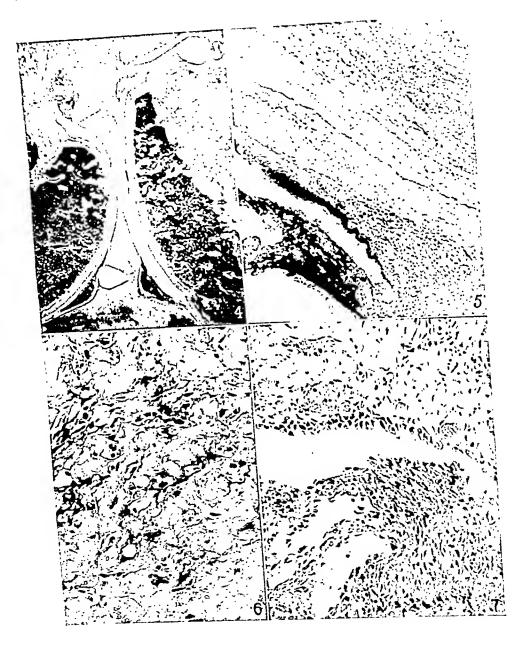
PLATE 12

Fig. 12, a. Focus of fibrinoid degeneration in the interstitial tissue of the heart. Acute scurvy plus infection. This is one of several such foci in the heart of this animal. Note the thickening and brilliant eosinophilic staining reaction of the abnormal collagen. ×225.

Fig. 12, b. Joint recess beneath patellar tendon. Subacute scurvy plus infection. Drawing of lesion illustrated in Fig. 5. Note the tongue-like projection of brilliant eosinophilic material extending into the joint space. Strands and masses of a similar material extend into the subsynovial tissue. Note also the hyperplasia of the synovial lining. ×56.

Fig. 12, c. Part of the subcutaneous nodule illustrated in Fig. 9. Portions of the collagen take a brilliant eosinophilic stain resembling that of fibrin or hemoglobin. ×225.

PLATE 10
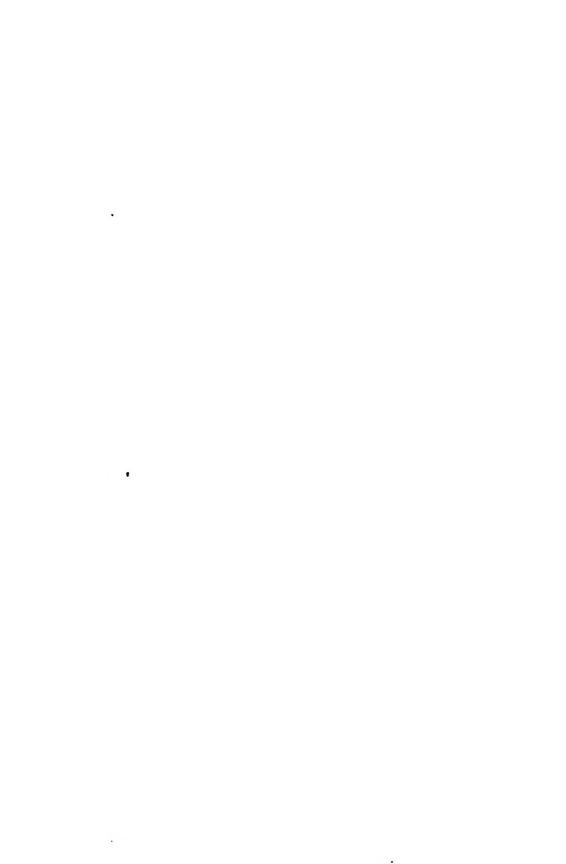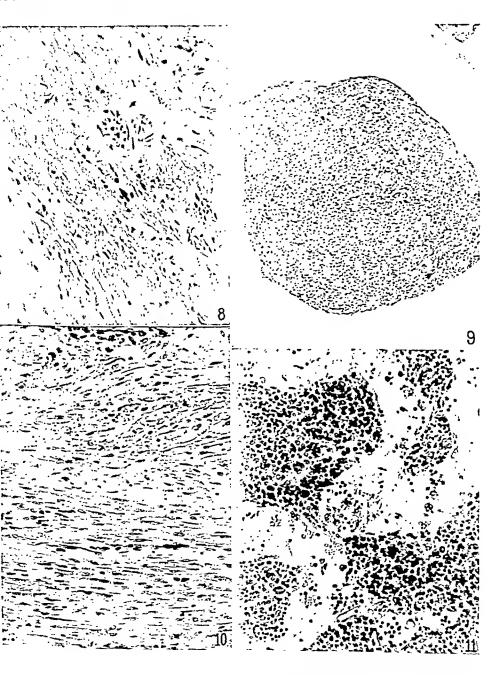
(Rinehart *et al.*: Scurvy with infection, and rheumatic fever)

ACUTE ASCENDING MYELITIS FOLLOWING A MONKEY BITE, WITH THE ISOLATION OF A VIRUS CAPABLE OF REPRODUCING THE DISEASE

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PLATES 13 TO 15

It is well known that acute ascending myelitis is not a single disease; it is a syndrome, which is characterized chiefly by rapidly ascending paralysis and high mortality and which may occur in the course of various forms of acute myelitis. Of the so called infectious myelitides, those which follow a host of various bacterial and virus diseases consti-tute the more frequent types, and are generally referred to as secondary or postinfectious myelitis. Pathologically this form of myelitis is characterized by perivascular demyelinization. Its pathogenesis and etiology are still obscure. Primary infectious myelitis is almost un-known, with the exception of rare cases of the so called spinal form of epidemic encephalitis, of acute anterior poliomyelitis which involves the remainder of the cord, and of certain recently described cases of acute ascending myelitis in which rabies virus is the probable cause (1). The purpose of the present communication is to describe, firstly, a fatal case of acute ascending myelitis which followed the bite of an apparently normal monkey, and, secondly, the isolation from the brain and cord and from the spleen of that case of a filtrable virus which reproduces the disease in rabbits.

History.—Dr. W. B., 29 years old, was engaged in experimental work on polio-myelitis. On Oct. 22, 1932, he was bitten on the dorsum of the left ring and little fingers at the terminal phalangeal joints, by an apparently normal *Macacus rhesus* monkey. The wounds, which were superficial, were painted with iodine and then with alcohol, and Dr. B. continued his work. The monkey died under ether

during an operation; no pathological examination was made. 3 days later, Dr. B. noticed pain, redness, and slight swelling at the sites of the bites. A lymphangitis developed and soon there was enlargement and tenderness of the left epitrochlear and axillary lymph nodes. In the afternoon of Oct. 28, he was admitted to the Third Surgical Service of Bellevue Hospital. His temperature was 101.4°F., the pulse was 90; physical examination revealed only the superficial redness and slight induration over the dorsum of the terminal phalanges of the left little and ring fingers with an associated regional lymphangitis and epitrochlear and axillary lymphadenitis. On the day of admission he received a prophylactic injection of tetanus antitoxin. In the course of the next few days he appeared to improve considerably; several small vesicles, containing a small amount of cloudy fluid, formed at the sites of the bites; the vesicles were opened on Oct. 30. The lymphangitis disappeared; the regional lymph nodes diminished somewhat in size and were only slightly tender.

On Nov. 1, 7 days after the first signs of infection of the fingers appeared, he developed generalized abdominal cramps, which lasted for 2 days and were not associated with tenderness, rigidity, nausea, vomiting, or diarrhea. On Nov. 4, he developed marked hyperesthesia of the lower extremities associated with urinary retention. Physical examination at that time revealed a generalized hyperalgesia below the level of the umbilicus; the knee jerks and abdominal reflexes were absent; the ankle jerks and cremasterics were present. The Babinski sign was negative; there were no signs of meningeal irritation; the upper extremities were not involved. At his own request he was given 20 cc. of convalescent poliomyelitic serum. The following day, Nov. 5, there was flaccid paralysis of both lower extremities. A spinal puncture performed that day yielded a clear fluid under slightly increased pressure with no evidence of block. Microscopic examination showed 112 cells per c. mm., all monocytes, albumen +, globulin +, reducing body 75.9 mg. per 100 cc.; smears and culture of the fluid were negative. On Nov. 5, after a neurological consultation by Drs. F. Kennedy and E. D. Friedman, the latter made the following note: "Pupils and other cranials negative. Upper extremities normal. Paraplegia involving all the muscles from the costal arch downward. Abdominals not obtained. Knee jerks absent; ankle jerks—left greater than right. No Babinski—plantars ventral. There is a level at about D 7 to D 8 below which pain and temperature senses are diminished. Tactile and posterior column sense not seriously altered. Bladder retention. Findings are those of a ventral myelitic lesion at the level noted. The etiology is obscure although the recent infection is probably related to the spinal condition." During the next day, Nov. 6, the sensory level had ascended to D 3, the ankle jerks were still present, and the upper extremities remained normal. On Nov. 7, he complained of paresthesias in the upper extremities and Dr. Friedman made the following note: "Pupils, fundi, and other cranials negative except for a few nystagmoid jerks in horizontal plane. Upper extremities normal; biceps and triceps jerks present. No Horner syndrome. Breathing mechanism intact." By this time

the ulcer on the little finger had entirely healed and was covered by a scab, and the one on the ring finger had become filled with granulation tissue. That night the temperature rose to 104.8°F. The following morning, Nov. 8, the temperature dropped to 99°F., but the patient looked very ill and complained of pain in the upper extremities. During the course of the day, hiccoughing developed and the respirations became slow and irregular. During the evening the respiratory
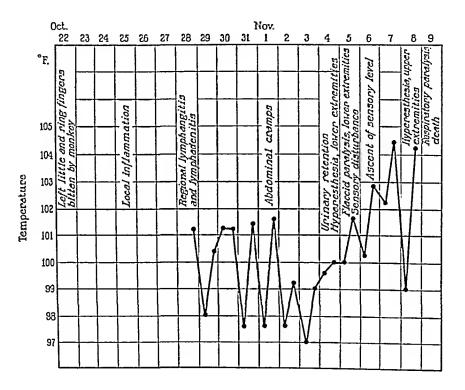
CHART 1. Clinical course of the human disease.

rate diminished to six a minute; he became quite cyanotic, and was put into a respirator. About 75 minutes later he had a convulsion, with apparently laryngeal spasm, and lost consciousness. Pulmonary edema developed, the frothy fluid being pumped out through the mouth and nose. Despite partial aspiration of the fluid, and the application of supportive measures, he lived only 5 more hours. (Chart 1.)

Results of Clinical Laboratory Procedures

(a) *Leucocyte Counts.*—

Date	Oct. 28	Oct. 31	Nov. 2	Nov. 5	Nov. 6
No. per c.mm. of blood	10,600	7,150	9,900	17,900	14,750
Polymorphonuclears, *per cent*	67	60	50	71	84
Metamyelocytes I, *per cent*			1	5	
Metamyelocytes II, *per cent*			15	6	
Monocytes, *per cent*	7	12	6	5	
Lymphocytes, *per cent*	26	26	25	13	16
Eosinophiles, *per cent*		2	3	0	

(b) *Blood Cultures.*—Blood cultures were taken on Nov. 4, 5, and 8; aerobic and anaerobic cultures were made and media suitable for growth of spirochetes were used. All were negative.

(c) *Lesions on Fingers.*—Direct smear revealed only a few cocci and many pus cells; dark-field examination was negative. Swab cultures yielded *Staphylococcus albus* (predominating) and *Streptococcus hemolyticus*.

(d) *Spinal Fluid.*—Cultures sterile; other findings already recorded.

(e) *Animal Inoculations.*—Two mice, two rats, two guinea pigs, and two rabbits were injected intraperitoneally with blood. One *Macacus rhesus* monkey was inoculated with the exudate from the lesion on the fingers (about 0.05 cc.) into the testicle, with 1 cc. of the spinal fluid intracerebrally and 0.5 cc. subcutaneously, and with 10 cc. of blood intraperitoneally. No significant results were obtained.

Abstract of Pathological Findings

Necropsy was performed by Thomas A. Gonzales, Deputy Chief Medical Examiner of New York City. Very few gross pathologic changes were found. In the left axilla there were several solitary enlarged lymph nodes the substance of which was moderately hemorrhagic. The spleen was rather soft in consistency; the pulp was deep red and the follicles were somewhat prominent. The urinary bladder extended midway to the umbilicus. The cortical convolutions of the brain appeared flattened. On section, the brain and the cord in the cervical, thoracic, and lumbar regions showed no gross lesions except that the cord was somewhat edematous.

The histological picture of the midcervical and upper dorsal regions of the spinal cord was that of an acute transverse myelitis. An inflammatory exudate of mononuclear cells, mostly perivascular, was present in the white matter as well as in the gray matter of the cord. The severest reaction in the gray matter appeared to be in the middle part and the base of the posterior horns. In the white matter of the cord there was a good microglial reaction but only a few of these cells had reached the stage of compound granular corpuscles. Sections stained for

neuroglia showed no proliferation of the astrocytes. Sections stained for myelin showed no tract degeneration in the spinal cord, nor any areas of perivascular demyelinization in the central nervous system. The medulla showed a marked inflammatory exudate, particularly severe in the floor of the fourth ventricle; this exudate was not mononuclear but rather predominantly polymorphonuclear with many large groups of cells suggesting abscess formation. The pons, the basal ganglia, internal capsule, and uncinate gyrus showed marked perivascular infiltration, mostly with round cells. The frontal lobe showed some small but quite definite hemorrhagic foci just beneath the surface. There was a mononuclear exudate about many of the vessels of the pia. The left brachial plexus showed no evidence of an inflammatory exudate nor of any myelin degeneration. (Figs. 1 and 2.)

Significant microscopic changes were observed also in the regional lymph nodes, the spleen, and the adrenals. Examination of the regional lymph nodes (left axilla) showed intense hyperemia and focal areas of hemorrhage. Beneath the capsule and for a short distance within the substance of the node, small foci of necrosis were seen. (Fig. 3.) Numerous sections stained by Twort, Gram-Weigert, and Ziehl-Nielsen methods, revealed no organisms. The spleen showed hyperemia and a few areas of necrosis similar to those seen in the lymph nodes. Sections through both adrenals revealed the presence of several large confluent necrotic areas, similar to those found in the lymph nodes and spleen. Those in the adrenals, however, were surrounded by a moderate number of leucocytes; bacterial stains revealed no organisms.[1] (Fig. 4.)

Clinical and Pathological Manifestations

Clinically there presented itself at the site of the monkey bites a mild and relatively insignificant cellulitis of the fingers which was followed by a mild regional lymphangitis and lymphadenitis. 13 days after the bite and 10 days after the first signs of local inflammation, there appeared the typical picture of a transverse myelitis which ascended and resulted in death from respiratory paralysis within 4 days. Clinically there were practically no manifestations pointing to an involvement of the brain. The pathological changes in the central nervous system were diffuse but most marked in the medulla and spinal cord. The predominance of mononuclear cells in the exudate, the perivascular round cell infiltration, and the other changes

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[1] The authors are indebted to Dr. Thomas A. Gonzales for the report on the gross pathology, to Dr. Lewis Stevenson for the report on the microscopic pathology of the nervous system, and to Dr. Irving Graef for that of the microscopic pathology of the other organs.

which were described, are pathological findings which are considered characteristic of virus diseases of the central nervous system in general, yet not characteristic of any virus disease in particular. It is only from the distribution of the pathologic process that one can perhaps exclude acute anterior poliomyelitis on a histopathologic basis alone; the absence of perivascular demyelinization which characterizes the so called acute disseminated encephalomyelitis, or postinfectious encephalomyelitis would serve to exclude this condition as well. The absence of any pathologic reaction in the regional brachial plexus, which would indicate the passage or the presence of the infectious agent, may have some bearing on the determination of the portal of entry of the infectious agent into the central nervous system and the pathogenesis of the disease. It becomes extremely important therefore to take note of the focal areas of necrosis which were observed in the regional lymph nodes, the spleen, and the adrenals. For the possibility, and forthcoming evidence, that these were caused by the same virus as that which injured the central nervous system, may not only elucidate the pathogenesis of this disease, but also serve to amalgamate these various organic changes into one pathological, and perhaps clinical, entity.

ISOLATION OF THE INFECTIOUS AGENT AND REPRODUCTION OF THE DISEASE IN ANIMALS

The material available for study consisted of pieces of brain, medulla, spinal cord, spleen, and regional lymph node which were obtained at necropsy 5 hours after death and preserved in 50 per cent glycerine. Although the microscopic pathology of the organs was unknown at the time this study was begun, the sterility of the blood and spinal fluid cultures taken during life, suggested the possibility that the causative agent belonged to the group of filtrable viruses. Since only a limited number of animals was available, portions of the brain, medulla, and spinal cord were pooled into one mixture and for most experiments the spleen and regional lymph node into another. 10 per cent emulsions of these pooled mixtures were prepared in the usual manner. Other emulsions prepared for different tests from tissue which had been in glycerine for periods varying from a few days to several weeks, proved unsterile on culture; direct

smear of the emulsions revealed no organisms but cultures on agar
and broth, or incubation of the saline emulsion itself, invariably
resulted in an almost pure growth of Gram-negative bacilli. Studies
of these bacilli indicated that they belonged to the colon group.
Macacus rhesus monkeys, dogs, guinea pigs, mice, and rabbits were
injected with the emulsions by various routes.

Transmission Experiments on Macacus rhesus Monkeys

Brain and Cord.—Monkey A received 2 cc. intracerebrally and 20 cc. intra-
peritoneally of a freshly prepared 10 per cent emulsion of the brain and cord.
Daily observations on the condition and temperature of the monkey revealed no
abnormal changes. The monkey died on the 11th day after injection. Post-
mortem examination of the brain and cord as well as of the other organs revealed no
significant findings; the histologic sections were unsatisfactory. Cultures taken
from the brain, cord, heart's blood, and peritoneum were sterile. The brain and
cord of this monkey were preserved in glycerine, and 8 days later a saline emulsion
was prepared from them and another *Macacus rhesus* monkey B was inoculated
intracerebrally and intraperitoneally; this monkey had fever on the 4th and 5th
days after injection, transitory slight tremors, but remained in good condition
over a period of 2 months' observation. In view of the fact that the results from
Monkey A were inconclusive, another *Macacus rhesus* monkey C was inoculated
intracerebrally and intraperitoneally with a freshly prepared emulsion of glycerin-
ated tissue of the original brain and cord. When Monkey C showed neither
fever nor any other abnormal signs for 3 weeks, it was reinoculated intracerebrally
and intraperitoneally with another freshly prepared emulsion of the original brain
and cord. This monkey remained well for 2 months after the last injection.

Spleen and Regional Lymph Node.—Monkey D was inoculated intracerebrally
(2 cc.) and intraperitoneally (20 cc.) with a freshly prepared 10 per cent emulsion
of the glycerinated spleen and regional lymph node; when after 2 weeks it failed
to show either fever or abnormal signs, it was reinoculated in the same manner
with another freshly prepared emulsion. Monkey D remained well.

From these tests it appears extremely unlikely that either the
brain and cord or the spleen and regional lymph node contained any
agent capable of inciting a specific disease in the *Macacus rhesus*
monkey.

Experiments on Mice, Guinea Pigs, and Dogs

Mice, guinea pigs, and dogs were injected intraperitoneally and intracerebrally
with emulsions of the original brain and cord, as well as of the spleen and lymph
node. No evidence of a virus disease was elicited in any of these animals, but

they succumbed to an infection with a Gram-negative bacillus present in the patient's organs, and the disease thus produced in no way resembled that from which the patient died. The organism was a member of the colon group, as already mentioned, and tests with it in pure culture showed it to be extremely virulent for mice, guinea pigs, and dogs. The bacteriological studies during life as well as the histopathological changes which were present in the patient's organs, leave little doubt that this organism was a postmortem contaminant.

Experiments on Rabbits

The transmission experiments on rabbits were carried out in much the same manner as for the other animals with the exception that the spleen and regional lymph node material were used separately.

Brain and Cord.—As indicated in Table I, of two rabbits (Nos. R 3-51 and R 3-52) which were injected intracerebrally with 0.5 cc. of the emulsion, one survived and the other died on the 3rd day. There were no suggestive signs before death; the postmortem cultures from the brain and heart's blood were sterile; the brain and cervical cord were somewhat congested. 0.5 cc. of a 10 per cent emulsion of the glycerinated brain and cord of the dead rabbit (No. R 3-53) was injected intracerebrally into another rabbit (No. R 3-64). Rabbit R 3-64 had excessive salivation on the 5th, 6th, and 7th days after injection but showed no other signs suggestive of encephalitis; 1 month after injection, this rabbit was retested with active virus. The results will be given elsewhere under the discussion of abortive infections and active immunity.
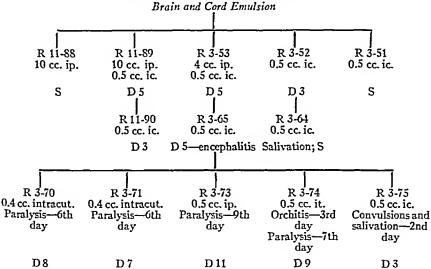
The one rabbit (No. R 11-88), injected intraperitoneally only, with 10 cc. of the original brain and cord emulsion, survived without signs. However, both rabbits (Nos. R 11-89 and R 3-53) which were injected intracerebrally as well as intraperitoneally died on the 5th day, again with sterile heart's blood and brain cultures, and also without any suggestive antemortem signs. As a result of later observations it is not improbable that these rabbits may have had antemortem convulsions but since the interval between the onset of these signs and death may be very short, these might not have been noticed. The brain and cord of each dead rabbit were glycerinated separately, and new rabbits were injected intracerebrally with fresh emulsions prepared from them. As shown in Table I both died—one on the 3rd day and one (No. R 3-65) on the 5th day. Rabbit R 3-65 was seen before death, and convulsions as well as excessive salivation were observed. The postmortem cultures of the heart's blood and brains of these rabbits were again sterile.

It seemed evident that the brains and cords of the rabbits that died as result of inoculation contained some virus which was capable of inducing an encephalitis and of being transmitted in series. The

question arose now whether or not it would be possible to reproduce the disease under investigation, by introducing the virus in a focus outside the central nervous system.

The glycerinated brain and cord of No. R 3-65 were used in these tests. Two rabbits (Nos. R 3-70 and R 3-71) were injected intracutaneously with a fresh 10 per cent emulsion, one rabbit (No. R 3-73) intraperitoneally, one (No. R 3-74)

TABLE I

Transmission Experiments in Rabbits with Human Brain and Cord

Brain and Cord Emulsion

R 11-88	R 11-89	R 3-53	R 3-52	R 3-51
10 cc. ip.	10 cc. ip.	4 cc. ip.	0.5 cc. ic.	0.5 cc. ic.
	0.5 cc. ic.	0.5 cc. ic.		
S	D 5	D 5	D 3	S
	R 11-90	R 3-65	R 3-64	
	0.5 cc. ic.	0.5 cc. ic.	0.5 cc. ic.	
	D 3	D 5—encephalitis	Salivation; S	

R 3-70	R 3-71	R 3-73	R 3-74	R 3-75
0.4 cc. intracut.	0.4 cc. intracut.	0.5 cc. ip.	0.5 cc. it.	0.5 cc. ic.
Paralysis—6th day	Paralysis—6th day	Paralysis—9th day	Orchitis—3rd day Paralysis—7th day	Convulsions and salivation—2nd day
D 8	D 7	D 11	D 9	D 3

In Tables I and II, ip. indicates intraperitoneal; ic., intracerebral; it., intratesticular; intracut., intracutaneous; D 5, dead on 5th day after injection; S, survived.

intratesticularly, and still another (No. R 3-75) intracerebrally. The rabbit injected intracerebrally developed convulsions 2 days later and died on the 3rd day. All the other rabbits, however, developed paralysis of the posterior extremities after a definite incubation period as indicated in Table I; this paralysis progressed more or less rapidly to involve the anterior extremities, the rabbits dying of respiratory failure without convulsions and with salivation occurring only immediately before death or not at all. A more detailed description of the course of events will be given in another part of this paper. The resemblance of the course of this disease in rabbits to that which was observed in the patient was most remarkable.

TABLE II

Transmission Experiments in Rabbits with Human Spleen

Spleen Emulsion

R 11-S7
6 cc. ip.

S

R 3-60
4 cc. ip.
0.5 cc. ic.
Paresis + tremors—3rd-11th days
Prostrate 12th day

D 13

R 3-67
0.5 cc. ic.
0.25 cc. intracut.
Convulsions and salivation—5 days

D 6

R 3-76
0.4 cc. intracut.
Paralysis—5th day

D 7

R 3-77
0.4 cc. intracut.
Paralysis—6th day

D 7

R 3-79
0.4 cc. intracut.
Paralysis—Sth day

D 9

R 3-S3
0.4 cc. intracut.
(Emulsion—1 mo, old)
Paralysis—6th day

D 7

R 3-72
0.5 cc. ic.

D 3

R 3-S0
0.4 cc. intracut.
(Supernatant liquid)
Paralysis—10th day

D 11

R 3-62
0.4 cc. intracut.
(Supernatant liquid)
Paralysis—10th day

D 11

R 3-99
0.2 cc. intracut.
0.5 cc. it.
Paralysis—5th day
Sacrificed

R 3-85
0.4 cc. intracut.
(Berkefeld V filtrate)
Paralysis—16th day

D 17

R 3-S6
0.5 cc. ic.
(Berkefeld V filtrate)
Encephalitis—4th
day

D 5

R 3-94
0.2 cc. intracut.
Paralysis—9th day

D 10

R 3-97
0.2 cc. intracut.
Paralysis—Sth day

D 10

R 9-01
0.4 cc. intracut.
Paralysis—6th day

D 6

R 9-02
0.2 cc. intracut.
Paralysis—Sth day
Sacrificed—Sth day

R 9-03
0.2 cc. intracut.
Paralysis—6th day

Spleen.—Table II reveals that the transmission experiments with the original spleen emulsion yielded a virus which behaved in precisely the same manner as that which was shown to be present in the original brain and cord.   It will again be noted that in the first passage intraperitoneal injection alone failed to give results whereas the combined intracerebral and intraperitoneal injection resulted in a take which permitted of ready subsequent transmission.

The first passage rabbit (No. R 3-60) manifested no characteristic signs and lived for 13 days; the second passage rabbit (No. R 3-67), however, injected with the brain and cord of No. R 3-60, developed typical signs of encephalitis on the 5th day and died on the 6th.   The postmortem smears and cultures of the heart's blood and brains of these rabbits were sterile.   The intracutaneous injection of a 10 per cent emulsion of this brain and cord (No. R 3-67) resulted in the typical development of first, paralysis of the posterior extremities with rapid progression cephalad and death by respiratory paralysis, without convulsions and little or no salivation.   The brains and cords of these rabbits were again capable of reproducing this disease in precisely the same manner when injected intracutaneously.

By these experiments a virus, transmissible in series, was shown to be present in the human spleen, as well as in the brain and cord, and capable of inducing a disease in rabbits, clinically similar to the human disease.   This virus has undergone fifteen serial passages now, and from the second passage onward has behaved like a fixed virus.

Regional Lymph Node.—The attempt to isolate a virus from the small piece of the regional lymph node which was available proved unsuccessful.   One rabbit injected intracerebrally with the emulsion died on the 6th day without any signs other than diarrhea.   The postmortem cultures were sterile, but another rabbit injected with an emulsion of its brain and cord survived 1 month without any signs.

Nature of the Virus

For the purpose of reference in subsequent discussions, the virus isolated from spleen, brain, and cord of the human case, will be called the B virus.

(a) *Disease Produced in Rabbits Following the Introduction of the B Virus by Various Routes*

Intracerebral Route.—The course of the experimental rabbit disease following the introduction of the virus directly into the brain differed considerably from that

which followed its inoculation into foci outside the central nervous system. During the first 48 hours after the intracerebral injection of 0.5 cc. of a 10 per cent fresh emulsion of glycerinated brain and cord, the rabbit appears entirely normal and the temperature shows no abnormal variation; within the next 12 to 24 hours the temperature may or may not rise and in rapid succession there appear generalized convulsions, increased salivation, and death.

Intracutaneous Route.—The routine procedure consisted of injecting 0.2 cc. of the 10 per cent brain and cord emulsion into two places (0.4 cc. altogether) on one
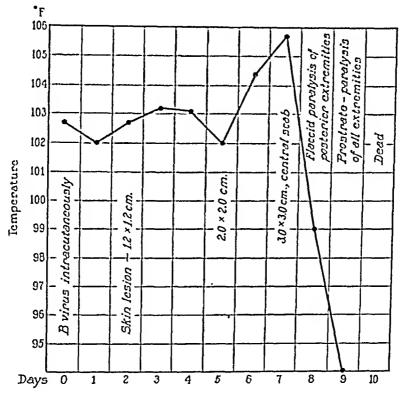
CHART 2. Temperature of rabbit injected intracutaneously with B virus.

side of the abdomen or back from which the hair was clipped. There was almost no skin reaction in the first 24 hours. Within the next 24 hours erythematous papules varying from 1 to 2 cm. in diameter appeared at the site of inoculation. The following day there was usually some hemorrhagic necrosis in the center of the papule which became more marked for another day and then proceeded to heal. About the 6th day after injection there was usually a rise in temperature (not always observed, however) and paralysis of one or both of the posterior extremities appeared. The side which was paralyzed first bore no relationship to the side receiving the intracutaneous injections. Within 12 to 24 hours the paralysis usually progressed cephalad to involve the fore limbs. The rabbit either died

within 24 hours of the onset of paralysis or lingered on in a prostrate condition with slow, gasping respirations (sometimes only once or twice a minute) for another 24 hours; but thus far, not a single rabbit which developed paralysis has survived. With one exception of uncertain nature, the paralysis which followed the intracutaneous injection of the virus was always flaccid and was not associated with convulsions. In some of the rabbits there was twitching of the facial muscles or convulsive movements of the head and slight salivation just before death; and in some a relaxation of the sphincters with almost continuous dribbling of urine was associated with the paralysis. In view of the fact that postmortem examinations revealed a markedly distended urinary bladder in most rabbits, it is highly probable that the dribbling of urine may be due to a relaxation of the sphincter secondary to urinary retention. (Chart 2.)

The resemblance of the disease produced in rabbits to that observed in the human case is most striking. A local, relatively insignificant, cutaneous lesion is followed after an interval by flaccid paralysis of the hind limbs associated apparently with urinary retention, and there is a cephalad progression of the process and death by respiratory failure.

Intratesticular Route.—The course of the disease which followed the intratesticular injection of the B virus can be best illustrated by a protocol.

Protocol of Rabbit R 3-74.—Inoculation: 0.5 cc. of 10 per cent emulsion of rabbit brain and cord (2nd generation passage virus) into left testicle.

Jan. 21, 1933. 103.5°F. Just before injection.

Jan. 22. 102.2°F. No apparent reaction.

Jan. 23. 103.0°F. No apparent reaction.

Jan. 24. 106.3°F. Marked swelling of left testicle.

Jan. 25. 103.2°F. Brawny induration of left testicle.

Jan. 26. 105.2°F. Testicle very hard and brawny; about three times natural size.

Jan. 27. 105.0°F. Same.

Jan. 28. 104.1°F. 2 p.m. Diminution in size of swollen testicle. Tremors and partial paralysis of right posterior extremity. 11 p.m. Complete flaccid paralysis of both posterior extremities; partial paralysis of right anterior extremity; cannot get up; no salivation.

Jan. 29. 95.6°F. 1:30 p.m. Condition same; paralysis definitely flaccid. Loss of sphincter control—feces and urine dripping continually. No salivation; slight retraction of head.

Jan. 30. Temperature unobtainable. Prostrate; gasping for air—only occasional breath. Lived on thus all day; slight salivation towards end. Died at night.

It will be seen from the protocol that the intratesticular injection of the virus resulted in an orchitis associated with fever on the 3rd day, and the development of typical flaccid paralysis on the 7th day. Although the virus was injected into the left testicle, it was the right hind limb which was paralyzed first. An ascending myelitis, rather than an encephalitis, invariably followed the intratesticular injection of the B virus.

Intraperitoneal Route.—For 8 days following the intraperitoneal injection of 0.5 cc. of the same virus emulsion into a rabbit no abnormal changes either in the temperature or physical status could be observed. On the 9th day there was a rise in temperature and in the evening of that day partial paralysis of both posterior extremities was present. On the 10th day the temperature had dropped to a subnormal level, and there was complete paralysis of the posterior extremities and loss of sphincter control (postmortem examination revealed a markedly distended urinary bladder); there was no salivation. Death occurred on the 11th day with evidence of antemortem salivation.

Corneal Route.—All attempts to implant the virus on the cornea of rabbits proved unsuccessful. Emulsions of the original brain and cord (human) were used as well as passage virus. There was neither local keratitis nor any demonstrable systemic invasion. It will perhaps be interesting to note here that whereas intracutaneous injection of the virus always caused the disease, there was no apparent reaction to virus introduced on the scarified skin. All the rabbits which were used for the corneal implantation tests were subsequently proved to be susceptible to the virus.

(b) Pathology of the Experimental Disease

The gross pathology of all the dead rabbits was recorded as routine and in a few selected instances the microscopic pathology was studied. Necropsy on twelve rabbits which died following intracerebral inoculation of the virus showed no obvious gross pathological change other than slight to moderate congestion of the brain and the cervical portion of the spinal cord. In contrast to the findings in the rabbits which died following intracutaneous injection of the virus, no lesions were discernible in any of the abdominal viscera. The rabbits which were injected intracutaneously, intraperitoneally, and intratesticularly showed, in addition to the congestion of the spinal cord and brain, the following grossly apparent changes in the abdominal viscera. The spleen was enlarged to twice or three times its usual size in almost every case; in many instances, grayish white spots, about 1 to 2 mm. in diameter, were seen just beneath the capsule and on section in the central portion of the parenchyma. In a few rabbits there was a congestion and mottling of the adrenals visible in the gross, and lesions were found with the microscope in other adrenals that appeared normal. The liver,

in many instances, showed the same grayish white spots that were observed in the spleen. In approximately 50 per cent of the rabbits the urinary bladder was found to be markedly distended. No pathologic changes were observed in the heart, lungs, and kidneys.

The evaluation of the microscopic changes produced by the virus in the central nervous system of rabbits, proved to be difficult on account of the frequency with which lesions characteristic of *Encephalitozoon cuniculi* were encountered. The brains and cords of three rabbits which died following intracutaneous injection of the virus showed no *Encephalitozoon cuniculi* lesions; in these there was no perivascular cellular infiltration but instead a pericellular and perivascular edema of the type which Brown and Symmers (2) described in certain human cases which they called acute serous encephalitis. There was no meningeal reaction. The most prominent change was present in the spinal cord and consisted of neuronic damage as evidenced by varying degrees of necrosis, hyperchromia, vacuolization, nuclear degeneration, and by a slight, rather diffuse infiltration with mononuclear cells. Definite characteristic inclusion bodies of the type observed in the other organs were not encountered, but various types of intranuclear bodies were seen in the large cells of the anterior and lateral horns. Stains for myelin revealed no foci of demyelinization. The paucity of microscopic lesions may perhaps be explained by the fact that only 24 to 48 hours intervened between the onset of neurologic signs and death. (Figs. 5 to 7.)

Striking microscopic changes were observed in the skin, adrenals, spleen, liver, and injected testicle. Sections through the skin lesion (at the site of inoculation), stained with hematoxylin and eosin, showed extensive necrosis of the cutis and subcutaneous tissue with a moderate infiltration of polymorphonuclear and mononuclear cells. One Giemsa-stained section of a skin lesion (tissue obtained after death) showed typical eosinophilic intranuclear inclusion bodies in the epithelial cells at the periphery of the lesion (Fig. 13). One of the skin lesions was excised 48 hours after injection of the virus; careful search of the Giemsa-stained section failed to reveal any inclusion bodies although definite necrosis was present. Characteristic areas of necrosis of varying size were found in all the adrenals examined except those obtained from a rabbit which was injected with the virus intracerebrally. The areas of necrosis were found chiefly in the cortex and were not surrounded by a zone of cellular infiltration (Fig. 8). Examination of Giemsa-stained sections revealed many typical eosinophilic intranuclear inclusion bodies in the cortical cells surrounding the necrotic zones (Fig. 9). The spleen showed focal necrosis with a few intranuclear inclusion bodies in endothelial cells (Fig. 12). The liver similarly showed numerous foci of necrosis (Fig. 10); eosinophilic intranuclear inclusion bodies were found in liver cells and although more numerous than in the spleen, they were not as abundant as in the adrenals (Fig. 11). The injected testicle showed widespread necrosis and cellular infiltration but insufficient work was done on the demonstration of inclusion bodies. The intranuclear inclusion bodies which were observed in the organs mentioned differed in no way from those described for herpes, varicella, Virus III disease, or salivary gland disease of guinea pigs.

The chief pathologic manifestations of the experimental virus disease in rabbits present many features in common with those in the human disease. The occurrence of focal necrosis in the adrenals and spleen in addition to central nervous system involvement is a striking feature in both. But although the visceral lesions resemble each other strongly, the pathologic picture in the central nervous system is not precisely the same. However, the difference of species or the shorter duration of the experimental disease may perhaps be responsible. In experimental poliomyelitis in the monkey, the microscopic pathology may vary with the virulence of the virus—the more virulent virus producing a less intense inflammatory reaction and a more rapid death.

(c) Filtrability of the B Virus

Although the failure to demonstrate any microscopically visible organisms either directly or on ordinary culture media in the tissues and fluids of rabbits dying from the experimental disease, and the successful transmission of the disease in series, together with the production of typical intranuclear inclusion bodies, left little doubt that the causative agent can be grouped with the so called filtrable viruses, it was nevertheless important to determine its filtrability.

Some of the "filtrable viruses" pass through the ordinary bacteriological filters with great difficulty or not at all. Ward and Tang (3) demonstrated that by emulsifying herpes virus in broth instead of saline, the virus could be recovered consistently from a Berkefeld V filtrate. The emulsions for the present tests were prepared in broth of pH 7.6. Two sets of tests were performed: one with a Seitz filter and the other with a new Berkefeld V candle, using negative pressure in each case. The period of filtration with the Seitz filter was 3 minutes, and with the Berkefeld V candle, 40 seconds; the broth emulsion was centrifuged at high speed and the supernatant liquid was used for filtration as well as for the control inoculations.

The results of the tests are shown in Table III. The rabbit injected intracerebrally with the Seitz filtrate failed to develop any signs. The filtrate from the Berkefeld V candle, however, produced the typical experimental disease, both on intracutaneous and intracerebral inoculation. The increased incubation period, however, suggested that there was less virus in the filtrate than in the highly centrifuged

supernatant liquid from which it was derived. The Berkefeld V
filtrate showed no growth on ordinary culture media; the same candle

TABLE III

Filtrability of B Virus

Material tested	Type of filter	Rabbit No.	Route of inoculation	Dose	Results
				cc.	
3rd generation passage virus. 10 per cent emulsion of brain and cord in broth	Unfiltered supernatant liquid	R3-79	Intracutaneous	0.4	Skin lesion—48 hrs.; paralysis—8th day; dead—9th day
	Seitz	R3-78	Intracutaneous	0.4	No skin lesion or paralysis; died—12th day of intercurrent infection
	Seitz	R3-80	Intracerebral	0.5	Excessive salivation and spasticity—12th, 13th, and 14th days; recovered; reinoculated 33 days after injection
4th generation passage virus. 15 per cent emulsion of brain and cord (No. R3-79) in broth	Unfiltered supernatant liquid	R3-80	Intracutaneous	0.4	Practically no skin lesion; paralysis—10th day; dead—11th day
	Unfiltered supernatant liquid	R3-62	Intracutaneous	0.4	Skin lesion—6th day; fever and increase in skin lesion—7th, 8th, and 9th days; paralysis—10th day; dead—11th day
	New Berkefeld V	R3-85	Intracutaneous	0.4	No skin lesion; paralysis—15th day; dead —16th day
	New Berkefeld V	R3-86	Intracerebral	0.5	Fever, paresis and complete paralysis of left anterior extremity— 4th day; salivation and death—5th day

was used subsequently for the filtration of pneumococcus broth
cultures and yielded sterile filtrates.

(d) "Viability" of Saline Suspensions of the Virus

Since most viruses deteriorate rather rapidly when not preserved in glycerine or by other dehydrating procedures, it is important to record the fact that a 10 per cent saline emulsion of a third generation passage virus (rabbit brain and cord) produced the typical disease upon intracutaneous injection just as well and with the same incubation period after the emulsion had been in the ice chest at 5°C. for 1 month, as it did when freshly prepared. This test was originally performed with the idea that the virus might be sufficiently attenuated by this procedure to permit its use in immunity experiments.

(e) Abortive Infections and Active Immunity

It is characteristic of most virus diseases that one attack, abortive or otherwise, imparts immunity to the surviving animal. The study of the immune phenomena with the B virus was important not only for itself but as an aid to establishing its identity and relationship to other known viruses.

Two rabbits had what may perhaps be called abortive attacks. One (No. R 3-80) was injected intracerebrally with a Seitz filtrate of an active virus emulsion and developed only spasticity and excessive salivation on the 12th, 13th, and 14th days but recovered completely; but upon reinoculation intracutaneously with active virus 33 days after the first injection, it developed typical paralysis and death occurred even though no skin lesion resulted at the site of inoculation. The other (No. R 3-64) was injected intracerebrally with a brain and cord emulsion of first passage virus (No. R 3-52) which produced only excessive salivation on the 5th, 6th, and 7th days; but 1 month after the first injection it was reinoculated intracerebrally with active virus, developed typical signs of encephalitis, and died. Another rabbit which after scarification of the cornea had virus introduced into the conjunctival sac on several occasions and failed to develop keratitis, succumbed in a typical manner after intracutaneous injection of active virus. Rabbit R 3-51 which failed to develop any signs after the intracerebral injection of the original (human) brain and cord, later succumbed typically to the intracutaneous injection of active virus.

In appraising these results it must be remembered that not a single rabbit which developed the typical experimental disease survived. More work will be necessary with graded doses of virus in the attempt to produce non-fatal attacks as well as in the testing for acquired

resistance, before any definite statement can be made about the development of immunity.

Relation of the B Virus to the Known Viruses

During the course of the present study the question naturally arose as to whether the B virus was a strain of an already known virus or whether it had not hitherto been described. The fact that the original material failed to take in *Macacus rhesus* monkeys and dogs whereas a characteristic disease was reproduced in rabbits, would seem to exclude the viruses of poliomyelitis and rabies as we know them. They can also be excluded by the type of inclusion body which the B virus induced and the absence of Negri bodies in its case. The necrotic lesions which were found in the adrenals in the present study are very like the lesions in the adrenals produced experimentally by the viruses of vaccinia (4) and herpes (5). The typical intranuclear inclusion body produced by the B virus as well as the absence of the Guarnieri bodies of vaccinia, would appear to limit the known possibilities to those viruses which give rise to the same type of inclusion body; *i.e.*, herpes, varicella, Virus III disease of rabbits, salivary gland disease of guinea pigs, and so called visceral disease (6). Of this group of viruses only two need be considered; namely, Virus III disease (a spontaneous disease of rabbits) and herpes. Although Virus III must be considered and guarded against whenever the experimental reproduction of any disease is attempted in rabbits, the circumstances under which the B virus was isolated, the regularity with which the human organs repeatedly infected rabbits from different sources, the striking similarity of the experimental and human disease, as well as the dissimilarity with that which Virus III is known to induce, all point against its identity with the B virus. The exclusion of Virus III disease leaves only the virus of herpes for consideration. Certain strains of herpes encephalitis virus resemble the B virus in the following respects: (*a*) localization in the central nervous system following intracutaneous injection, (*b*) morphologically similar necrotic lesions in the adrenals following intracutaneous injection, and (*c*) similar intranuclear inclusion bodies. There are almost as many differences between the two, however: (*a*) although the herpes virus may produce myelitis when injected into zones supplied by nerves which enter the

spinal cord (7), it produces an encephalitis primarily when injected intratesticularly (7-9), whereas the B virus invariably attacks the spinal cord first, as evidenced by the flaccid paralysis of the posterior extremities and urinary retention, with cephalad progression of the lesion and death by respiratory failure, (b) to cause focal necrosis of the spleen and liver a direct injection of herpes virus must be made into these organs (7), whereas these lesions are a part of the systemic disease following the intracutaneous injection of the B virus, (c) in the present study it has been impossible to produce keratitis with the B virus. The final determination of the identity or distinctness of the two viruses must depend upon cross-immunity tests. Unfortunately, no rabbits have recovered from infection with the B virus thus far nor has an immune rabbit been secured by any method attempted. Attempts to immunize rabbits to intracerebral inoculations with virulent herpes virus in order to test them for immunity to the B virus have been unsuccessful. However, it would appear that the B virus possesses certain characteristic properties which justify its consideration as a distinct entity.[2]

SUMMARY

A case of acute ascending myelitis which followed the bite of an apparently normal *Macacus rhesus* monkey is described. The clinical course as well as the pathological changes has been studied and found to be suggestive of a virus cause for the disease. The absence of perivascular demyelinization removes the case from the realm of acute disseminated encephalomyelitis and establishes it more or less definitely as a primary acute infectious myelitis. An extremely important feature of the pathological picture of this disease has been the presence of focal necrosis in the viscera (spleen, adrenals, regional lymph nodes).

Attempts to transmit the disease to *Macacus rhesus* monkeys, dogs, mice, and guinea pigs, employing glycerinated organs from the human

[2] Through Dr. Josephine B. Neal, Dr. Gay and Dr. Holden obtained from us some brain and cord from the human case. In a preliminary paper (*Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 1051, Case 4) they report the demonstration of a virus having the properties of our B virus as here described and state their belief on experimental evidence that this virus is identical with the herpes virus.

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case, proved unsuccessful. By the inoculations of rabbits the presence of a strongly neurotropic, filtrable virus was demonstrated in the patient's brain, cord, and spleen. Following intracutaneous injection of it as derived either from brain and cord or spleen, an experimental disease develops in rabbits which strikingly resembles the human disease in the character of the local lesion, the incubation period, development of urinary retention, and flaccid paralysis of the posterior extremities with cephalad progression, death by respiratory failure, and finally by the occurrence of focal necrosis in the spleen, adrenals, and liver. In attempting to establish the identity of this virus, (the B virus), a consideration of its biological properties excludes the viruses of poliomyelitis, rabies, vaccinia, Virus III disease of rabbits, and the other viruses which are known to produce similar intranuclear inclusion bodies, except perhaps herpes. Although the relationship between the B virus and the virus of herpes must still be determined by cross-immunity tests it has been shown to possess certain properties which warrant consideration of it as a distinct entity.

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EXPLANATION OF PLATES

PLATE 13

FIG. 1. Cross-section of human spinal cord showing a large area of necrosis; section stained for myelin, Loyez method. × 7.

FIG. 2. Human spinal cord showing extensive cellular infiltration, particularly in white matter; note necrotic state of some of the nerve cells. × 135.

The authors are indebted to Dr. Lewis Stevenson for the photographs shown in Figs. 1 and 2.
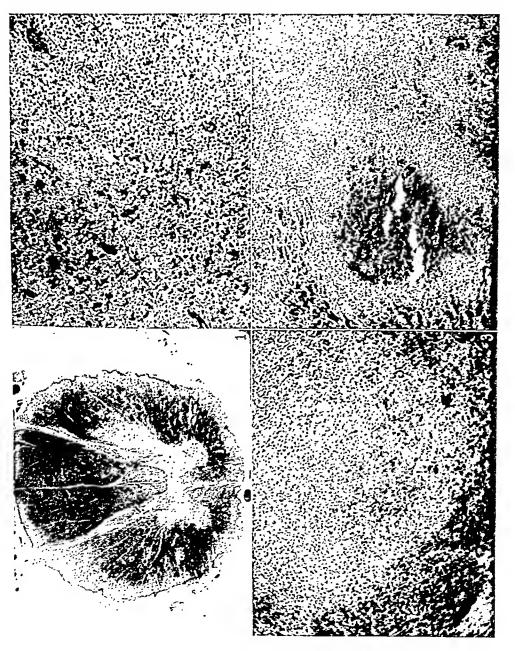
FIG. 3. Human axillary lymph nodes showing focal necrosis. Hematoxylin and eosin. × 135.

FIG. 4. Human adrenal showing large necrotic area surrounded by zone of cellular infiltration. Hematoxylin and eosin. × 135.

PLATE 14

FIG. 5. Spinal cord of rabbit injected with B virus intracutaneously; large ganglion cell showing vacuolization, beginning neuronophagocytosis, and nuclear degeneration. Hematoxylin and eosin. × 1,160.

FIG. 6. Same as Fig. 5. Zenker fixation and Giemsa stain. Arrow points to dark red intranuclear body (not a typical inclusion body). × 1,160.

FIG. 7. Same as Fig. 5. Note invasion of gray matter by mononuclear cells and anterior horn cells in various stages of necrosis. Hematoxylin and eosin. × 145.

FIG. 8. Adrenal of rabbit injected with B virus intracutaneously; note large area of necrosis. Hematoxylin and eosin. × 145.
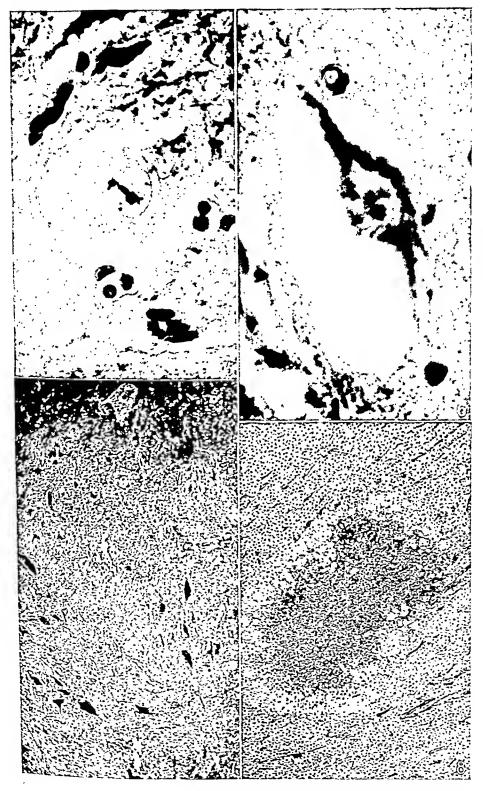
PLATE 15

FIG. 9. Same as Fig. 8. Zenker fixation and Giemsa stain. Zone surrounding area of necrosis in adrenal; arrows point to nuclei containing eosinophilic inclusion bodies. × 1,210.

FIG. 10. Liver of rabbit, injected intracutaneously with B virus, showing focal necrosis. Hematoxylin and eosin. × 150.

FIG. 11. Same as Fig. 10. Arrow points to nucleus of liver cell containing eosinophilic inclusion body. Hematoxylin and eosin. × 1,210.

FIG. 12. Spleen of rabbit injected with B virus intracutaneously; note focal necrosis. Giemsa. × 600.

FIG. 13. Section of rabbit skin at site of inoculation with B virus; Zenker fixation and Giemsa stain. Almost all the epithelial cells in this zone contain eosinophilic intranuclear inclusions. × 1,210.
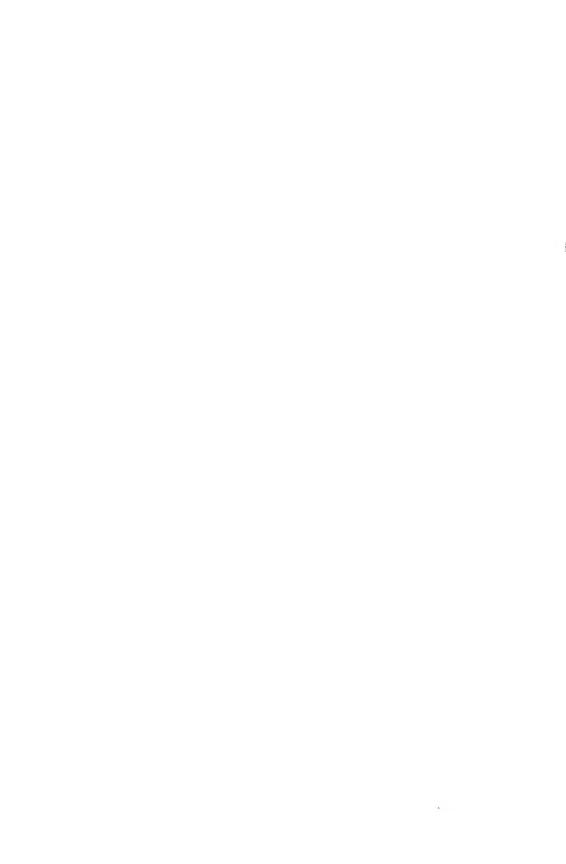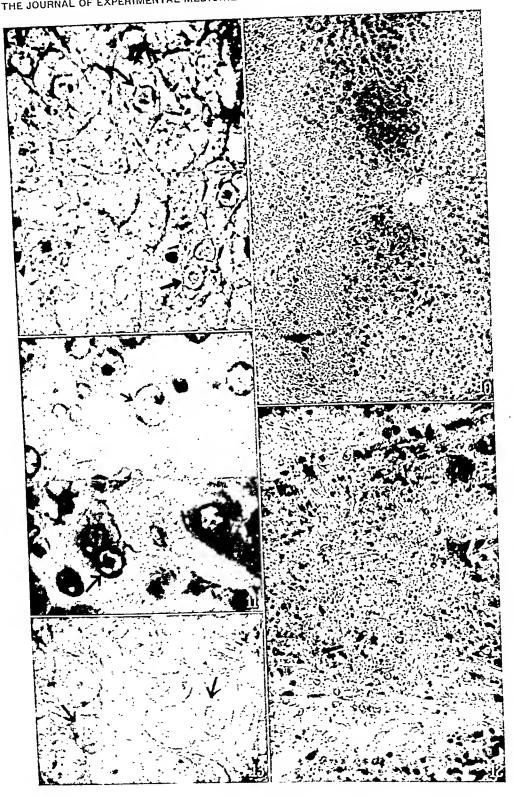
(Sabin and Wright: Isolation of virus reproducing myelitis)

PLATE 15

EXPERIMENTS ON THE CONVERSION OF TYPHUS STRAINS

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It is a well established fact that the strains of Mexican typhus which have been established in laboratory animals during recent years differ in several points from all the well studied strains of Old World typhus, in spite of complete cross-immunity between the two kinds of strains. Regular scrotal swelling in guinea pigs with numerous rickettsiae in the tunica vaginalis, and a febrile, often fatal disease in rats with numerous rickettsiae in the tunica, distinguish Mexican strains clearly from Old World strains of typhus. Old World strains produce scrotal lesions only rarely, the lesion as a rule is mild, and rickettsiae can be found in the animals only with great difficulty and very irregularly. In rats they are said to cause inapparent infections only. Since our Mexican strains isolated in previous years from man show all the characteristics of strains obtained from wild rats in nature, we call them murine strains, whether they have been isolated from rats or from man. Recently we were able to isolate from a long standing epidemic of typhus several strains which correspond closely to strains of Old World typhus. We call them epidemic strains. We avoid the word endemic for those strains which cause scrotal lesions in guinea pigs and fever in rats for two reasons: first, we were able to isolate from short lived, rapidly checked epidemics in Mexico City only strains which are in every respect identical with strains obtained from isolated endemic cases and from rats in nature; second, from isolated cases in Europe only typical strains of Old World or epidemic typhus have been reported so far. The finding of typical epidemic strains in Mexico where previously only murine strains have been isolated by us, seems to be in agreement with the hypothesis of Nicolle that there must exist in Mexico two varieties of typhus, the Mexican variety of murine origin and the historic (1) louse-borne

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typhus imported from abroad. We, however, are of the opinion that the murine strains represent the original form of the virus, whereas the epidemic strains are the product of a secondary adaptation by a long standing transmission in the cycle man-louse-man. The transformation of murine strains into epidemic strains is not amenable to an experimental demonstration. The reverse, however, namely the transformation of epidemic non-orchitic strains into strains which show the murine characteristics, can be tried in the laboratory. The present paper deals with experiments which show that the properties which characterize the murine strains are contained in latent form in the epidemic strains, and that by proper handling the original murine traits become clearly manifest in the epidemic strains.

Material and Methods

The murine strains do not require any description on this occasion. They have been described in earlier papers (2) and are entirely identical with strains of endemic typhus in the southern United States, with strains isolated from rats and rat fleas by Dyer and his associates (3), from rats and rat fleas in France, Greece, and Syria (4), and with strains of so called Machurian typhus (5). Our epidemic strains, however, deserve a detailed description because they are the first non-orchitic strains of typhus which we were able to establish in Mexico.

In 1932 an epidemic of typhus started among an Indian tribe in the State of Oaxaca. This epidemic spread slowly northwards among the native population along their paths of trade. In February, 1933, when the epidemic had assumed alarming proportions among the Mixtec Indians in the Sierra of Oaxaca and in several large Aztec Indian settlings in the adjoining parts of the State of Puebla, we decided to visit these regions with the aim of isolating virus strains from a long standing, serious epidemic. We were especially interested in finding out whether strains from such a long standing epidemic in Mexico corresponded to the murine type of the virus as did those isolated from short lived, rapidly checked epidemics in the capital, or whether they corresponded experimentally to strains of historic (1) Old World typhus. The conditions we met there were typical of epidemic typhus. The Indians were covered with head and body lice and in many huts we found two to four patients ill with severe typhus. In others we found one or several acutely ill, with other members of the family lying around as convalescents. Many of them were mourning one or several members.

Five strains of virus were established: one from the blood of a patient in Chila, three from blood of various patients in Zinacatepee, and one from lice collected

from a patient on the 10th day of the disease also in Zinacatepec, in the State of Puebla. White rats, as well as guinea pigs, were used for isolating the strains. Each material was inoculated into two or three rats, and into two or three guinea pigs. The superiority of the rat was revealed by the fact that of the four blood strains obtained, all four were established from rats and only one in addition from guinea pigs. The louse strain was obtained both from guinea pigs and rats. In all, blood samples from six patients in the 1st week of illness were inoculated into the animals, giving a positive take in rats of 66.66 per cent of the cases, and a positive take in guinea pigs of 16.6 per cent. 15 days after inoculation, the rats which had not shown any sign of illness during that time were killed, and the strains established in guinea pigs by inoculation of brain emulsions. The louse strain showed scrotal swelling in guinea pigs in the first three transfers, with rickettsiae in the tunica. Then the phenomenon disappeared for several months entirely, but recently has been appearing again in an occasional transfer guinea pig. On no occasion was the lesion so pronounced as we are accustomed to find in our previously isolated murine strains. Blood Strain 2 showed a transient scrotal reaction in the first passage in guinea pigs, and since February, 1933, this symptom has appeared twice again. The other three blood strains never produced the slightest scrotal involvement. In guinea pigs all five strains caused high continuous fever of from 8 to 9 days' duration, after an incubation period lasting from 7 to 8 days.

Histological examination of the brains revealed nodular lesions with great regularity. In rats the two strains which caused occasional scrotal involvements in guinea pigs produced frequently a mild short fever without any other clinical symptoms; whereas the three other strains ran in rats a purely inapparent course, and correspond therefore exactly to strains of historic Old World typhus in respect to their experimental behavior. No rickettsiae could be found in rats inoculated with these strains. All five strains, however, as could be expected, showed complete cross-immunity to our murine strains, and rat to rat transmission was easily accomplished with *Xenopsylla cheopis*.

The experiments were carried out with these five epidemic strains. The method employed for their transformation is based on the following observations.

In examining sections of lice and fleas infected with the virus of typhus, it is observed that *Rickettsia prowazeki*, the causative agent, multiplies only within the epithelial cells of the mid-gut; *i.e.*, in cells which come in frequent contact with fresh blood. Never has there been observed in insects the slightest invasion of the muscular apparatuses, the genital organs, the fatty tissue, nor of the salivary glands. Only in fleas the lower parts of the Malphigian tubules near their union with the gut are frequently found to be invaded by *Rickettsia prowazeki*. When guinea pigs and rats are inoculated subcutaneously, *Rickettsia prowazeki* develops in the endothelial cells of the blood vessels only. Absolutely never does it invade cells which lie beyond the endothelial lining of a blood vessel. When animals are

inoculated intraperitoneally with a murine strain, *Rickettsia prowazeki* multiplies abundantly within the endothelial cells of the tunica vaginalis. Examination of guinea pigs killed during the incubation period revealed that infected endothelial cells of the tunica vaginalis are found only where the serosa covers superfically situated blood vessels, especially along those vessels which are situated in the groove separating the fatty body from the testicle proper. Such places seem to constitute the primary site of multiplication of *Rickettsia prowazeki* in the tunica vaginalis. When a guinea pig with a fully developed scrotal reaction is killed, the infected serosa cells are found to be distributed over the entire tunica vaginalis. At that time the scrotal sac is found to be filled with coagulated blood or plasma.

It seems evident from these observations that in the mammal, as well as in the insect, *Rickettsia prowazeki* is in some way dependent for its intracellular development on the presence of fresh blood which has to come in close contact with the susceptible cells. Why the rickettsiae of Old World typhus and of our recently established epidemic Mexican strains show so little tendency to grow in the processus vaginalis of guinea pigs and especially of rats where the rickettsiae of the murine variety multiply so abundantly, is a problem yet to be solved. A working hypothesis was, however, conceived which seems to have brought us a step forward in the understanding of the conditions involved. Since in murine strains scrotal lesions in guinea pigs occur regularly, whereas they are the exception in epidemic strains, we thought that the rickettsiae of the murine strains were less hemophilic than the rickettsiae of the epidemic strains, being able to multiply in the processus vaginalis when a transudation of a small quantity of blood or plasma occurs through the superficially situated blood vessels on account of intraperitoneal inoculation of foreign material. The ensuing specific inflammation on the sites of the primary multiplication of *Rickettsia prowazeki* along the superficially situated blood vessels causes a more or less abundant exudation of plasma which collects in the scrotal sac and creates favorable conditions for the spreading of the organism over the entire endothelial lining of the processus vaginalis. For the rickettsiae of the epidemic strains, which apparently are much more hemophilic than those of the endemic strains, the conditions for growth in the peritoneal cavity are not favorable. To be able to multiply within the endothelial cells they seem to need a steady and abundant supply of fresh blood, a condition present only in blood vessels. With this idea in mind, we decided to inject daily fresh blood into the peritoneal cavity of

guinea pigs and rats previously inoculated by the same route with the virus of the non-orchitic epidemic strains. Fresh guinea pig blood, whole or defibrinated, was used in nearly all of the experiments. Since in the endemic strains multiplication of rickettsiae takes place almost exclusively in the tunica of guinea pigs and rats, we included an endemic strain in our experiments in order to find out whether this method produced in rats a general infection of the endothelial lining of the peritoneal cavity. Full grown white rats were used in all experiments.

Experiments with a Murine Strain

Two rats were inoculated intraperitoneally with tunica and testicular washings from a guinea pig killed when scrotal swelling was at its height. Immediately after and from then on every morning the rats received 3 cc. of whole guinea pig blood intraperitoneally. A control rat received the same amount of blood every day. When the inoculated rats looked very ill they were killed and smears made from the visceral and parietal peritoneum. Both rats showed an extensive invasion of the peritoneal cells with *Rickettsia prowazeki*. The majority of the cells were found to be crowded full with organisms and very numerous extracellular rickettsiae were spilled from disintegrated cells. Protocol 1 illustrates the course of fever in these animals.

Experiment I.   Protocol 1

	Rat 1 ♀	Rat 2 ♀	Control ♀
	°C.	°C.	°C.
July 15 inoculated	B 37.2	B 37.3	B 37.1
July 16	B 37.1	B 37.3	B 37.4
July 17	B 37.4	B 37.5	B 37.3
July 18	B 38.2	B 38.1	B 37.5
July 19	B 38.2	38.3	B 37.4
July 20	B 38.1	Looks ill, killed. Rickettsiae ++++. Spleen very large. A large amount of brownish smeary exudate covering peritoneum. Few coagula	B 37.6
	Ill, killed. Rickettsiae ++++. Spleen very large. Much brownish exudate in peritoneum. Few coagula		

± = Very few infected cells.
\+ = Few infected cells.
++ = About 10 per cent cells infected.
+++ = Between 20 to 50 per cent cells infected.
+++ + = The majority of cells infected.
B = Blood injection.

This experiment was remarkable inasmuch as it was conducted with females in which rickettsiae can as a rule be found only with difficulty. In no instance did we see such large numbers of rickettsiae in the peritoneal cavity of rats which had been inoculated directly from a guinea pig. Only x-ray-treated rats may show such a heavy infection of the peritoneal cavity (6). From the peritoneal exudate of Rat 1 three new rats were inoculated. Two of them received a daily blood injection; the third served as an infected control without blood.

Experiment II.   Protocol 2

	Rat 4 ♂	Rat 5 ♀	Rat 6 ♂
	°C.	°C.	°C.
July 20 inoculated	B 37.2	B 37.2	37.3
July 21	B 37.4	B 37.4	37.2
July 22	B 37.4	B 37.5	37.3
July 23	B 38.4	B 38.5	38.4
July 24	B 38.3	B 38.6	38
July 25	B 38.2	B 38.5	38.1
July 26	B 38	Died in the evening.	36.2
July 27	B 37 Looks very ill, killed. Rickettsiae, tunica +. Rickettsiae, peritoneum ++. Spleen very large. A good deal of exudate	Rickettsiae, peritoneum +++. Spleen very large	Dying. Rickettsiae, tunica +. Rickettsiae, peritoneum ±. Spleen very large

Two more transfers were made from rat to rat with and without additional blood injections. In both instances the rats died between the end of the 3rd and the end of the 4th day with heavy rickettsiae infection of the peritoneal cavity. Although in the animals injected with blood considerably more rickettsiae were found, in the animals not injected with blood the infection had also spread over the entire peritoneal cavity in the last transfer.

Experiments with the Epidemic Strains of Mexican Typhus

A. Epidemic Blood Strain 1.—Experiments were carried out first with a strain which, since its isolation, had never shown the slightest

scrotal involvement in guinea pigs, nor had frequent examinations of the tunics of transfer animals ever shown any rickettsiae. This strain constantly showed numerous typical brain lesions in guinea pigs. In rats it caused an inapparent infection only when brain of transfer guinea pigs was inoculated, and on no occasion did we find rickettsiae in such rats.

A transfer guinea pig received a daily blood inoculation intraperitoneally. On the 7th day this animal showed fever and a typical scrotal swelling was observed, for the first time in this strain, with very numerous rickettsiae in the tunica. Tunica and testicular washings from this animal were inoculated into two white rats followed by a daily injection of 3 cc. of guinea pig blood. Two male guinea pigs were also inoculated with the same material without consecutive blood injection. Both guinea pigs ran a typical non-orchitic course characteristic of this strain. Protocol 3 shows the course of infection in two rats.

Experiment III. Protocol 3

	Rat 7 ♂	Rat 8 ♀
	°C.	°C.
July 18 inoculated	B 37.3	B 37.2
July 19	B 37.3	B 37.4
July 20	B 37.7	B 37.8
July 21	B 37.3	B 37.6
July 22	B 38.6	B 39
July 23	38.8	B 38.5
	Sick; killed. Rickettsiae, tunica ++++. Rickettsiae, peritoneum ++++. Spleen very large	Looked sick in the evening, killed. Rickettsiae, peritoneum ++++

From Rat 7 three male guinea pigs were inoculated intraperitoneally with peritoneal exudate, and two male rats with the same material. The latter received a daily guinea pig blood injection, whereas the guinea pigs did not receive any blood. All three guinea pigs reacted with fever after an incubation period of 3 days only, and in all of them a typical scrotal lesion appeared between the 4th and 5th day. Protocol 4 illustrates the course of the disease in these two rats.

Protocol 4

	Rat 9 ♂	Rat 10 ♂
	°C.	°C.
July 23 inoculated	B 37.3	B 37.4
July 24	B 37.4	B 37.5
July 25	B 37.5	B 37.6
July 26	B 38	B 38.1
July 27	36.2	B 38
July 28	Very ill, killed. Rickettsiae, tunica ++++. Rickettsiae, peritoneum +++++. Extremely numerous extracellular rickettsiae, giving the impression of a culture. Spleen very large	B 36 Looked ill that morning. Found dead in the evening. Spleen very large. Very numerous badly staining rickettsiae in tunica and peritoneum

From Rat 9 three male guinea pigs and two female rats were inoculated and the rats treated with daily blood injections. The three guinea pigs developed fever after an incubation period of 3 days only, and typical scrotal involvement appeared in all of them. The rats showed fever on the 3rd day and were moribund on the 4th with heavy rickettsiae infection of tunica and peritoneum.

After a few more transfers through rats, the strain, which previously had caused only inapparent infection in rats, killed them now regularly between the 4th and 5th day, with or without the application of blood. In this strain also repeated transfers from rat to rat caused a diffuse invasion of the peritoneal cavity with rickettsiae even when no blood was injected, but a heavy infection could as a rule be found in those animals only in the tunica; whereas in the blood-treated animals a heavy rickettsiae infection was found over the whole peritoneal cavity.

The experiments with this typical non-orchitic epidemic strain demonstrated clearly that by the blood method the strain assumed characteristics found hitherto only in endemic murine strains; *i.e.*, scrotal lesions in guinea pigs and a fatal course of disease in rats, with numerous rickettsiae in the peritoneal cavity.

B. Epidemic Louse Strain.—The same procedure was followed with this strain as that which was applied to the epidemic blood Strain 1; namely, inoculating a transfer guinea pig with a daily dose of blood. No scrotal lesion appeared, but the tunica was found to

be edematous and hemorrhagic on the 1st day of fever although no rickettsiae could be found. Tunica emulsion and testicular washings from this animal were inoculated into rats, followed by a daily dose of 5 cc. of blood. One rat died on the 6th day from peritonitis. Two others had fever on the 8th and 9th days; blood injection was discontinued on the 9th. The next day when the rats were very ill they were killed, and numerous rickettsiae were found in the tunica and in the peritoneum. From one of these animals transfers were made to other rats and the strain then became highly virulent following the third transfer, with enormous numbers of rickettsiae in the blood-treated animals. In guinea pigs inoculated with peritoneal exudate of such rats, scrotal lesions appeared with great regularity; but when transfers were made then from guinea pig to guinea pig, the strain rapidly reverted to its non-orchitic original course.

C. *Blood Strains 2, 3, and 4.*—Strains 2 and 3 could also be adapted to rats with the blood method. A highly fatal disease resulted in these animals, with the same enormous invasion of the peritoneal cavity by rickettsiae. The inoculation from such rats into guinea pigs regularly caused a scrotal involvement, but on further transfers from guinea pig to guinea pig the strains rapidly reverted to their original non-orchitic course. Both these strains were adapted to rats by inoculation of guinea pig brain followed by blood injections, instead of using tunica as was done in the previous experiments. The epidemic blood Strain 4 behaved differently from the other epidemic strains inasmuch as we have not yet been able to convert it. On no occasion did it cause fever in rats and only exceptionally could a few rickettsiae-infected cells be found in the peritoneal cavity of animals infected with this strain.

Experiments with Nicolle's African Strain of Historic Typhus

This well known strain which we owe to the courtesy of Professor Charles Nicolle, of Tunis, has been kept in our laboratory since July, 1931. In transfer guinea pigs this strain causes as a rule only a rare and transitional scrotal reaction. Occasionally, however, a lesion may be observed which looks exactly like that observed in our murine strains. When transfers into rats are performed and brains of such rats inoculated back into guinea pigs, scrotal lesions may

appear in nearly 50 per cent of these animals; but on further transfers
from guinea pig to guinea pig the lesion becomes rare again.   When
brain of guinea pigs infected with this strain is inoculated into rats,
no fever is observed and no rickettsiae can be found in the peritoneal
cavity.   The disease remains entirely inapparent.   When, however,
tunica exudate from a guinea pig with scrotal involvement is inocu-
lated into rats, a short fever may be observed occasionally, and rickett-
siae, although as a rule not numerous, are found in the tunica of such
rats.   It is this strain which Professor Nicolle has compared with
one of our Mexican rat strains, coming to the conclusion that the
two types of strains behave entirely differently in rats (1).   He admits
for his strain only a purely inapparent infection in rats as he does for
all Old World strains.   That this is not entirely the case we have
already demonstrated (7).   On no occasion, however, had we ob-
served in this strain previous to the present experiments a serious
course of the disease in rats even when they had been inoculated
with a large amount of tunica from an occasional orchitic guinea pig.
Another difference between the murine strain and the Tunisian strain
to which Nicolle gives much importance is his observation that while
the Mexican murine strain can be carried indefinitely in transfers
from rat to rat, the Tunisian strain breaks off sooner or later in such
rat transfers.   Nine successive transfers in rats were the longest series
that he was able to obtain (8).   Since Nicolle's strain is one of the
best studied Old World viruses, we thought that it would be of
particular significance if we succeeded in transforming this strain
into a strain highly pathogenic for rats with the characteristics of our
Mexican murine strain.

Two separate strains of Nicolle's virus which assumed murine characteristics
with our blood method were established.   One strain was started from the tunica
of a guinea pig with scrotal involvement, and the other from the brain of a guinea
pig without scrotal involvement.   The guinea pig from which the first strain was
started had been inoculated with brain of a rat and was then injected daily with
5 cc. of guinea pig blood.   On the 7th day, when the guinea pig showed a sug-
gestive scrotal lesion, it was killed and the tunica in which few rickettsiae were
found was inoculated into rats.   These rats received a daily dose of 3 cc. defi-
brinated guinea pig blood.   The rats did not show any signs of illness, but when
killed on the 5th day typical infected endothelial cells were found in the tunics.
By further transfers from rat to rat using tunics and 3 cc. of guinea pig blood,

typical fever curves appeared in the rats, and in the animals of the third transfer enormous numbers of rickettsiae-infected cells were observed in the tunica smears. In the general peritoneal cavity, however, they were scarce or absent. We increased, therefore, the daily blood dose to 5 cc. of whole blood every day in the succeeding transfers. Most of the rats so treated now died around the 5th day, with a general heavy rickettsia infection of the whole peritoneal cavity.

Infections were as severe as those found in our Mexican strains, and frequently almost 100 per cent of endothelial cells were found to be crowded full of rickettsiae. Inoculation from such heavily infected rats into guinea pigs caused pronounced scrotal swelling in the great majority of them, and in rats it caused a serious fatal disease, often without fever. After several transfers with the blood method, the strain became so virulent that an additional injection of blood was no longer necessary. Some rats showed paralysis of the hind legs shortly before death, and extensive specific lesions were found in the medulla oblongata of these rats. The second strain which behaved entirely analogously was obtained by inoculating brain and blood of a guinea pig into several rats and then making transfers with the blood method from rat to rat.

Protocols 5 and 6 give the course of rats of the sixth and seventh transfers of Nicolle's strain.

Protocol 5

	Rat 132 ♂	Rat 133 ♂	Rat 134 ♂
	°C.	*°C.*	*°C.*
Aug. 21	Inoculated	Inoculated	Inoculated
Aug. 22	37.3	B 37.2	B 37.4
Aug. 23	37.3	B 37.4	B 37.3
Aug. 24	36.6. Ill	B 38.5	B 36.8. Looks ill
Aug. 25	36.4	36.3	36.5
	Hind legs paralyzed, killed	Looked ill during a.m.	Very ill, killed
Aug. 26	Spleen enlarged. Rickettsiae, tunica ++. Rickettsiae, peritoneum ±	Found dead in evening. Spleen very large. Rickettsiae, tunica +++. Rickettsiae, peritoneum ++++	Spleen very large. Rickettsiae, tunica ++++. Rickettsiae, peritoneum ++++

Protocol 6

	Rat 137 ♂	Rat 138 ♂	Rat 139 ♂
	°C.	°C.	°C.
Aug. 23	Inoculated	Inoculated	Non-infected blood control
Aug. 24	B 37.3	B 37.2	B 37.3
Aug. 25	B 37.2	B 37.1	B 37.1
Aug. 26	B 39.2	B 38.5	B 37.3
Aug. 27	B 38.2	B 38.3	B 37.4
Aug. 28	35.3. Very ill	35.5. Moribund	B 37.2
	Killed. Rickettsiae, peritoneum +-+-+. Rickettsiae, tunica, mostly naked nuclei of endothelial cells. Very numerous extracellular rickettsiae	Killed. Rickettsiae, peritoneum +-+-+-+, most cells in disintegration. Rickettsiae, tunica mostly naked nuclei. Very numerous extracellular rickettsiae	Killed. Normal appearance of organs. No microorganisms in smears. Some coagulated blood in peritoneal cavity

In guinea pigs the inoculation of peritoneal exudate of such rats regularly produced scrotal lesions as already mentioned. With the addition of blood the scrotal lesion appeared in nine successive guinea pig transfers. In guinea pigs not inoculated with blood, the strain reverted rapidly to its original non-orchitic course. The strain was kept in rats through fifteen successive transfers when the experiment was discontinued on account of lack of rats. No diminution of virulence was observed in the fifteenth transfer, as three of the four rats of the last transfer died at the end of the 5th day. The fourth rat recovered in spite of a severe paralysis of the hind legs.

The Result of Prolonged Transfers in Rats of the Adapted Epidemic Strains

Whereas the adapted epidemic strains reverted rapidly to their original non-orchitic course when put back into guinea pigs after only a few transfers in rats, they assumed definite murine characteristics after prolonged transfers in rats. The inoculation of these strains back into guinea pigs from rats now regularly caused a scrotal lesion which did not disappear on further transfers in guinea pigs, and also in rats they now behaved constantly like our murine strains even

when brain of guinea pigs was inoculated. The same observation in respect to rats was made with the strain of Nicolle, although this strain ran a non-orchitic course in guinea pigs in most of the animals.

The Staining of Rickettsiae in Smears from Rats and Guinea Pigs

The best results are invariably obtained with Giemsa's stain. This method, however, has the disadvantage that on prolonged staining which is frequently necessary, very numerous reddish staining cellular debris are stained which makes the observation of extracellular rickettsiae difficult and sometimes impossible. Somewhat less of these debris are seen when the smears after fixation with methyl alcohol are treated during 5 minutes with ether, and then are washed again with methyl alcohol. Careful differentiation with alcohol-xylol as recommended by Nigg and Landsteiner (9) frequently gives good results. Castaneda's method (10), as well as Lépine's modification of it (11), demonstrate the intracellular rickettsiae very well as a rule, when a good brand of methylene blue or azure II is used. The extracellular rickettsiae, however, frequently stain very poorly with this method. Lépine's original method (12) does not stain rickettsiae at all. In rats which were examined many hours after death, rickettsiae as a rule stained very poorly and most of the endothelial cells were found to be autolyzed. This poor staining was as a rule accompanied by clumping of the intracellular rickettsiae into red granular masses simulating inclusion bodies in virus diseases. Also the extracellular rickettsiae examined many hours after the death of the rats were recognized only with difficulty because their form was ill defined and they stained much redder than usual.

DISCUSSION

The finding of epidemic strains of typhus in Mexico which correspond experimentally to strains of historic Old World typhus seems to support the ideas of those who hold that there exist two varieties of typhus in Mexico: one, the New World type derived from the rat reservoir in nature, and the other, the historic Old World, purely louse-borne type, imported from abroad. This hypothesis has indeed been advanced recently by Nicolle (1). His main reason for not accepting the hypothesis that Old World typhus may also be originally derived from rats is the following: (1) typhus existed in Europe before the continent had been invaded by rats; (2) the murine type of virus can be differentiated clearly from historic typhus by the different reactions which it induces in laboratory animals, especially in rats. Now it has been shown beyond doubt that the murine type of virus can be transmitted by the human louse (13) and that the virus of

epidemic Old World typhus can be transmitted experimentally from rat to rat by the rat flea (7). Moreover, from short rapidly checked epidemics of typhus in Mexico City we were never able to obtain strains which did not correspond entirely to the murine type of virus. The same type of virus was obtained constantly from isolated cases during interepidemic periods and from wild rats in nature (14). There cannot exist, therefore, the slightest doubt that the murine virus actually does cause epidemics, and we were able to show that the virus isolated from the brains of wild rats multiplies abundantly in the human louse (15). We have, then, this situation in Mexico: from rats, from endemic cases of typhus, and from cases during short epidemics, murine strains only could be isolated; whereas from a long standing serious epidemic, strains were obtained which correspond to strains of historic Old World typhus. But not all of our epidemic strains agree completely with Nicolle's strain of Old World typhus. Whereas two of them cause a mild but typical fever in rats, three of them induce only inapparent infections in that species. Immunologically however all of them are identical. Non-orchitic strains of typhus have, without doubt, been isolated previously, all of them in times of long standing serious epidemic, by Gavino and Girard (16), Anderson and Goldberger (17), and Olitsky, Denzer, and Husk (18). It was in Mexico, indeed, that the work of Nicolle and his collaborators on the infection of the guinea pig with typhus was first confirmed, and it is not conceivable that the conspicuous scrotal reaction characteristic of all murine strains should have escaped the observation of so many investigators.

The finding, during a serious epidemic of typhus, of epidemic strains which correspond exactly to strains of historic Old World typhus and of strains which experimentally lie between typical epidemic strains and orchitic murine strains is in our opinion of great significance. After long series of transfers in rats with our blood method, all these epidemic strains, including that of Nicolle, with one exception assumed the typical murine characteristics. In later transfers the addition of blood was not necessary to cause a serious disease in rats with plentiful rickettsiae in the peritoneal cavity. This abundant multiplication of rickettsiae in rats is absolutely characteristic of all strains of the murine type, and the transformation of the non-orchitic

epidemic strains into strains of high virulence for rats shows clearly that there does not exist any fundamental difference between the two types of strains. When the epidemic strains were put back in guinea pigs, after only a few passages in rats, they invariably reverted to the non-orchitic form. When, however, these strains were kept in long series of rat transfers, they were found to have become definitely murine. Only Nicolle's strain could not be kept definitely orchitic in guinea pigs after long rat transfers, although it showed a definite and apparently permanent reversion to the murine type inasmuch as it is now highly virulent for rats, a property which Nicolle (1) considers to be the only valuable characteristic in differentiating historic Old World typhus from the murine New World typhus. But even before the epidemic non-orchitic strains had been adapted fully to rats, their murine traits could be occasionally elicited. When brain of a guinea pig of such a strain is inoculated into rats, a purely inapparent infection follows. When, however, tunica of an animal with an occasional scrotal swelling is inoculated into rats, a fever of short duration appears frequently. Since scrotal swelling in Old World strains had not been observed before one of us had published his results with Mexican typhus (2, 3) the conclusions of European investigators were based on inoculations with brain emulsions. Now it is a well established fact that a scrotal involvement is accompanied by an enormous accumulation of the virus of typhus in the tunica vaginalis. It is evident, therefore, that fever or no fever in rats depends entirely on the doses of virus inoculated and so cannot be a specific sign for differentiating strains of the murine type from strains of historic typhus.

Our hypothesis that the rat is the natural original carrier of the virus of typhus, murine and epidemic, seems to be well supported by the result of our experiments. We consider the epidemic variety to be the product of a long standing propagation of the murine type in the cycle man-louse-man; and it is thus not astonishing, and in complete agreement with our hypothesis, that the rat is the proper experimental animal for reverting epidemic strains into murine strains. The mechanism which causes the reversion of epidemic strains to the murine type of strains we consider to be the result of progressive selection during the transfers from rat to rat. All epidemic strains contain the

murine properties in a latent form, because scrotal lesions in guinea pigs show up occasionally in them, and the injection of tunica emulsion from such orchitic animals may cause fever in rats and rickettsiae in the tunica. This we take to mean that epidemic strains contain in small number individual rickettsiae having the properties of murine strains. These are individuals endowed with recessive properties. In transfers from rat to rat with the blood method, these recessive individuals of rickettsiae become more and more numerous by progressive selection until the strain has reverted to murine. In guinea pig transfers, the individuals with murine properties may become suppressed again, and the strain then reverts to its epidemic form. That the murine strains contain an epidemic component of rickettsiae is evident from the observation which we have frequently made in past years that such strains may suddenly become non-orchitic for many generations. In one of our rat strains the orchitic component was lost nearly completely by prolonged transfers in guinea pigs. Inoculation of brain emulsions from such non-orchitic guinea pigs only rarely caused fever in rats, but transfers from rat to rat restored to the strain its original orchitic properties. Here again we see the ability of the rat to restore the murine qualities of the strain which had been lost in long transfers through guinea pigs, an animal which naturally is not a host of the typhus virus. A phenomenon is therefore observed in guinea pigs similar to that which can be observed during times of epidemic in Mexico. In long transfers through an unnatural host, in this case man, the original murine components are suppressed. One of our epidemic strains, in which the murine qualities could not be restored, seems to have lost its murine components altogether.

On various occasions one of the writers has pronounced the opinion that the human louse cannot be the natural vector of typhus, and man therefore is not the natural host. The louse is in the biological sense so little adapted to *Rickettsia prowazeki* that it succumbs invariably to the infection within a relatively short time. In hot weather an infected louse survives but a few days, whereas the rat flea as shown by Dyer and his associates (19) and by Mooser and Castaneda (20) remains definitely infected without apparent harm. This we take as definite evidence that the human louse has appeared on the scene of typhus relatively late and that it has not yet had

time to become a highly adapted biological vector. That Old World typhus can be transmitted indefinitely at least in cold countries by the human louse we are not in a position to question. What we do question seriously, however, is the opinion of European workers that this is the only means by which historic typhus is preserved. On several occasions we tried to infect lice on very mild cases of Mexican typhus without the slightest success. No lice became infected. The number of infected lice is in our experience in direct relation to the severity of a case of typhus. There is little chance for lice to become infected on cases which run a mild short fever, which can be diagnosed as typhus only with the help of the Weil-Felix reaction, and there can be no doubt that inapparent human infections are even less liable to infect lice. On account of these experimental results we question the great importance which Old World investigators give to inapparent infections as a reservoir of the virus during long interepidemic periods. There is in addition no reason to believe that during long interepidemic periods all cases of typhus should be inapparent. It seems to us more than likely that what is happening in Mexico must happen the world over; i.e., the epidemic adaptation of the original murine virus to the secondary unnatural cycle man-louse-man. The circumstance that the murine virus has also recently been found in Greece and in Syria where epidemics of typhus have occurred frequently, and the existence of endemic murine typhus in rats and man side by side with historic typhus in such a typhus-ridden country as Manchuria, is of great significance in this respect. Nicolle (1) made the statement that the murine virus kills lice in such a short time that epidemics caused by this type of virus are doomed to die out quickly. He states furthermore that the virus of historic typhus survives much longer in lice. We have no experience with Old World virus in lice, but we know from the literature that it kills them also within from 10 to 14 days. From experiments with typical murine strains (21) we know that the longevity of lice depends entirely on the number of rickettsiae ingested. When concentrated tunica exudate is introduced by the method of Weigl, the lice do indeed die within a few days. The more the infective material is diluted, however, the longer they survive. Lice fed on monkeys infected with a murine strain (21) and lice fed on human volunteers inoculated

with the same strain lived 10 to 14 days (22).   There does not exist, therefore, the slightest reason for rejecting the human louse as a vector in epidemic form of the murine virus.

That the rat constitutes a natural reservoir of endemic typhus in the southern part of the United States, a possibility clearly foreseen by Maxcy (23), has been demonstrated beyond the shadow of a doubt by Dyer and his associates (3).   This important discovery will go far to explain the origin of human typhus everywhere.   Indeed there is not a single circumstance which speaks against the hypothesis that the historic typhus of the Old World is also derived from the rat reservoir in nature.   That this is the case for epidemic typhus in Mexico we consider to be definitely demonstrated.   From short lived rapidly checked epidemics in Mexico, the murine virus was isolated in each instance; from a long standing epidemic, strains were isolated which correspond to the typical historic Old World type of virus and in addition other strains which lie intermediate between the two extremes.   Moreover the epidemic strains could be induced to assume all the characteristics of typical murine strains by long standing transfers through rats with our blood method.

The observation that *Rickettsia prowazeki* is able to develop in the mammal as well as in the insect only within those cells which come into constant contact with fresh blood explains why typhus cannot be transmitted by ticks and by mites which take blood only at long intervals.   It is therefore not astonishing that we were not able to confirm the results of Shelmire and Dove (24) with *Liponyssus bacoti*. The virus may occasionally survive in these mites as it does in ticks for several days, but never could we demonstrate any multiplication of it and on no occasion was transmission accomplished with mites or ticks by the act of biting.   *Rickettsia prowazeki* exhibits the most specialized type of parasitism which has yet been observed in a bacterial pathogenic organism.   Not only is it dependent on blood while in the mammal host, but it continues to be dependent on mammal blood during its life in the insect vector.   This dependency on mammal blood may explain to a certain extent why the virus of typhus exhibits so little specificity toward animals and toward blood sucking insects.   Practically all rodents are susceptible to typhus as well as man, the apes, and the monkeys.   Of the blood-sucking insects

Pediculus, Pedicinus, Polyplax, and all species of fleas so far tested are susceptible. All these insects take blood at frequent intervals, 2 to 3 times a day. A persistence of the virus and a probable multiplication of it has been observed in *Cimex lectularius* (25). Although fresh blood is necessary for the development of *Rickettsia prowazeki* within cells, the cells do not play a merely passive rôle in this respect. We were not able to cause a general infection of the peritoneal cavity of guinea pigs by the blood method. In tissue cultures and in the Maitland medium *Rickettsia prowazeki* multiplies only in endothelial cells and never, for instance, in fibroblasts. That plasma or fresh serum is also necessary in these cultures for the development of *Rickettsia prowazeki* has been shown by Nigg and Landsteiner (9). In fleas as well as in lice no multiplication occurs in the cells of the hind-gut which also come into contact with blood. It is interesting that in the intestines of insects the susceptible cells are those of the intestinal tract which are covered by the chitinous peritrophic membrane. In fleas this membrane is well developed, whereas in lice the structure is thin and delicate. One is inclined to suspect that the development of *Rickettsia prowazeki* in these cells is related in some way to the metabolism of chitosamine.

In a series of experiments not recorded in this paper, we tried to determine what blood constituent is responsible for the enormous increase of *Rickettsia prowazeki* in the peritoneal cavity of rats injected with blood. Tests were made with serum, with washed red cells, and with defibrinated blood of various animals. Only with defibrinated blood of guinea pigs did we get results comparable to those obtained with whole blood of guinea pigs. As a rule, however, whole blood gave better results. Horse blood, sheep blood, and beef blood were found to be far inferior to guinea pig blood. To exclude a non-specific action of guinea pig blood on rats, we should have tried experiments using rat blood. The limited number of full grown rats at our disposal prevented us from carrying out these experiments. The observation that *Rickettsia prowazeki* can survive and multiply only in cells which come in contact with fresh blood furnished the basis for our blood method. Our uniform good results with guinea pig blood, however, offer no definite proof that whole blood or a blood constituent is really the specific factor which enables the rickettsiae to grow

in the peritoneal cavity. Zinsser and his pupils have succeeded in causing an enormous multiplication of rickettsiae in rats by various methods. Their x-ray method especially gives excellent results as a rule. They were, however, not able by their methods to adapt the rickettsiae of an epidemic non-orchitic strain to the peritoneal cavity of rats. It is possible that the positive results obtained by Zinsser and Castaneda with murine strains are based on the same principles as our blood method. Intensive x-ray treatment increases permeability of the walls of blood vessels, with subsequent effusion of blood constituents. For murine strains, the increased permeability caused by x-ray treatment seems to furnish enough of these constituents to allow the rickettsiae to grow in the general peritoneal cavity. For the epidemic strains which are much more hemophilic, this method is inadequate. In our experiments with Nicolle's epidemic strain, it was necessary to increase the daily blood dose from 3 cc. to 5 cc. in order to bring about a generalized infection of the peritoneal cavity.

SUMMARY AND CONCLUSION

Two types of strains of typhus virus are observed in Mexico: first the murine type which is obtained from wild rats in nature, from isolated endemic cases, and from cases during short epidemic outbreaks, and second, the epidemic type of strains which is obtained from long standing serious epidemics. Some of these epidemic strains correspond entirely to strains of historic Old World typhus. Other strains which in their experimental behavior are intermediate between these two types of strains were isolated from the same epidemic. A method is described by which these epidemic non-orchitic strains can be converted into murine strains regularly causing scrotal lesion in guinea pigs and a highly fatal disease in rats. The same results were obtained with an Old World strain of epidemic typhus. The method, which consists of daily blood injections into intraperitoneally inoculated rats, is based on the observation that *Rickettsia prowazeki* multiplies only within cells which come in constant or frequent contact with fresh blood. It is concluded from our experiments that there does not exist any real difference between the virus of historic Old World typhus and the murine New World typhus. Both are considered to be of murine origin. The murine strains represent the

original form of the virus of typhus, whereas the epidemic strains are the result of a prolonged propagation in the cycle man-louse-man.

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COMPARATIVE STUDIES ON THE VIRUSES OF VESICULAR STOMATITIS AND EQUINE ENCEPHALOMYELITIS (1)

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Since Andervont and Theiler (2) induced encephalitis in white mice by means of intracerebral injection of the viruses of herpes and yellow fever respectively, these animals have been more widely employed by investigators for the experimental transmission of still other viruses.

In this paper we present an elaboration of earlier studies (3, 4) of the properties of vesicular stomatitis virus, and a similar investigation of the biological reactions of another virus inducing disease in horses, equine encephalomyelitis, in which the use of the mouse as a test animal has proved advantageous. We are indebted to Miss B. Howitt of the George Williams Hooper Foundation of the University of California for a specimen of the latter virus which was discovered by Meyer, Haring, and Howitt (5).

VIRUS OF VESICULAR STOMATITIS

The virus of vesicular stomatitis procured from horses and cattle and transferred to the pads of guinea pigs retains its dermotropism during continuous pad to pad passage (3, 4). As will be shown in this article, we have found that the virus also possesses neurotropic properties as determined by the results obtained through the employment of different routes of infection in white mice, guinea pigs, rats, rabbits, and monkeys.

Methods and Materials.—Two strains of vesicular stomatitis virus (Indiana and New Jersey) (1 a, 4) were available, samples being sent us through the kindness of Dr. W. E. Cotton of the United States Bureau of Animal Industry, who had propagated the strains in guinea pig pads for several years. Before use in our experiments, they were carried through twenty consecutive passages in the pads of guinea pigs. The material used as inoculum for animal or cultural tests con-

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sisted of a 1:10 suspension of virus-infected tissue in hormone broth at pH 7.5. Unless otherwise stated, the suspension was filtered through Seitz' discs and the filtrate shown to be bacteria-free by inoculation of leptospira medium and on blood agar incubated under aerobic and anaerobic conditions.

Effects on White Mice[1]

Intracerebral Injection.-- After intracerebral injection of 0.03 cc. of filtered vesicular stomatitis virus, irrespective of its origin, that is whether derived from affected pads of guinea pigs, brains of mice, rats, guinea pigs, and monkeys, or from tissue cultures, the mice succumbed rapidly and uniformly to fatal encephalitis. Within 30 to 40 hours after injection of either the Indiana or New Jersey strain, the animals exhibited pronounced hyperesthesia, ruffling of the hair, tremors, circus movements, ataxia, and weakness of the legs. The weakness usually progressed to spastic paralysis of the posterior extremities, associated with generalized involuntary muscular contractions. In the early passages death occurred within 48 to 72 hours after inoculation, but after fifteen consecutive brain to brain transmissions, the animals lived only 24 hours after inoculation. Blood obtained by means of cardiac puncture at the height of reaction failed in every instance to yield bacterial growth in culture medium.

The gross pathological changes consist of edema of the brain with congestion and an occasional, small, focal hemorrhage. The histopathological lesions are either absent in the meninges or noted as infiltrations, here and there, by a few monocytes. The brain itself exhibits general edema and inconspicuous, diffuse, monocytic reaction. The neurones reveal various stages of degeneration. The characteristic lesion is the pronounced necrosis of the Purkinje cells and also of the nerve cells of the motor nuclei in the brain stem. The spinal cord shows corresponding changes: the membranes are practically normal; the cord itself is edematous and many nerve cells are degenerated. While the nuclei of most neurones contain acidophilic granular material, inclusion bodies are not detectable. On the other hand, as we shall soon describe, mice infected by intranasal instillation of the virus, with a resultant more protracted clinical course, frequently reveal characteristic intranuclear inclusions. It is noteworthy that the infiltrative or productive lesions are less manifest in animals having a fulminating type of infection; the predominant change then is the extensive and marked destruction of neurones.

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[1] Throughout the experiments reported in this paper, the Rockefeller Institute strain of white mice was employed.

Ether anesthesia was used in all operations on animals.

We have observed more recently that while the dermotropic virus does not noticeably affect the kidney and liver of guinea pigs, neurotropic strains injure these organs in all mice and other experimental animals. Granular degeneration of the cells lining the renal tubules, especially those of the convoluted type, occurs to a greater or lesser degree. One also finds granular degeneration in the parenchyma of the liver and in more advanced cases, isolated, small areas of necrosis of liver cells and punctiform hemorrhages. The spleen and other organs are not, as a rule, affected.

Nasal Instillation.—Recently Webster and Fite have succeeded in transmitting a fatal encephalitis to white mice by means of nasal instillation of louping ill virus (6). Vesicular stomatitis virus also induces regularly a lethal encephalitic infection in white mice. This may be effected by the intranasal instillation of 0.04 cc. of filtrate by means of a tuberculin syringe fitted with a blunt needle, care being taken to avoid contact with the nasal tissues. The series of infections has been carried through twelve passages with the Indiana, and six with the New Jersey strain of the incitant, the brain of nasally infected mice being employed as inoculum in each transfer. All of fifty-four animals exposed to the virus in this manner succumbed within 5 to 8 days.

The symptoms of the infection are similar to those occurring after intracerebral injection, with the exception of a more prolonged incubation period, namely, 4 to 6 days. The pathological changes in the brain, cord, liver, and kidney are also similar, although inflammatory lesions, such as localized mononuclear infiltrations of varying degree, occur in the vessel sheaths and spaces, and as nodular accumulations in the gray and white matter of the central nervous system. The striking lesion consists of intranuclear inclusion bodies. From one to three or four such structures can be seen usually located in the nerve cells of the hippocampus and of the anterior gray matter of the cord. They are 1 or 2 microns in diameter, acidophilic, regular in outline, flat, refractive, and while they resemble the inclusions of Borna's disease, minute study reveals their difference from the latter. The bodies lie in sac-like nuclei which are somewhat swollen and have a darkly stained basophilic membrane, and are often in juxtaposition with the nucleoli. The most effective stain for their demonstration is phloxin-methylene blue (7).

It appears, therefore, that the virus of vesicular stomatitis is highly potent when applied to the uninjured nasal mucosa which is as sensitive to inoculation as is the traumatized brain or pads of animals. Infection was induced in white mice by this method with material

is associated with distinct, but not pronounced action on the parenchymatous tissue of the kidney and liver. In view of the high susceptibility of the uninjured nasal passages—as high in degree as is the traumatized brain—rigid precautions are necessary during experimental procedures to prevent exposure to accidental infection. The identity of the strains should be checked at monthly intervals by animal immunity tests.

Effects on Guinea Pigs

The results of inoculation of the pads of guinea pigs with the virus of vesicular stomatitis have already been described (3, 7, 9).

Intracerebral Injection.—After twenty-five consecutive transmissions of the experimental disease in pads, 0.15 cc. of filtrates of active plantar tissue was introduced intracerebrally in guinea pigs. The following symptoms were induced by both the Indiana and the New Jersey strains of the virus.

From 2 to 5 days after injection, the animals showed weakness which rapidly progressed to paresis of both posterior extremities. Tremors and circling movements were also observed. Within 2 or 3 days the posterior extremities exhibited complete flaccid paralysis, and about 75 per cent of the animals died during the paralytic stage. The survivors recovered partially; some showed only an ataxic gait, while others were left with paralyzed legs. After nineteen brain to brain passages had been obtained with the New Jersey and five with the Indiana strain of virus, the experiment was discontinued. The cerebral tissue derived from the second, fourth, and seventh passages was also inoculated, for control purposes, into the pads of guinea pigs, producing therein pronounced, characteristic vesicular dermatitis.

The gross and microscopic pathological changes in the brain, cord, kidney, and liver of guinea pigs which died of the experimental infection correspond with those observed in mice.

The guinea pig is susceptible to intracerebral injection of this virus, whether derived from guinea pig pad or brain; mouse, rat, and monkey brain, or tissue cultures (1 *b*). Infection was induced with these materials in dilutions up to 10^5.

Other Routes of Infection.—Pad inoculation of guinea pigs with plantar tissue virus with simultaneous intracerebral injection of 0.12 cc. of sterile starch solution induced no encephalitis, only the vesicular plantar dermatitis. Like mice, guinea pigs are unaffected by intraperitoneal inoculation either alone or with simultaneous intracerebral injection of starch solution.

Although the guinea pig is not as sensitive to the effects of the stomatitis virus in the central nervous system as is the white mouse.

the neurotropic action of the infective agent is nevertheless clearly demonstrated by the results of the foregoing experiments.

Effects on Monkeys

Macacus rhesus and *Macacus cynomolgus* monkeys were inoculated intracerebrally with 1 to 1.5 cc. of filtrates of 10 to 20 per cent suspensions of tissues containing vesicular stomatitis virus. The tissues employed consisted of (a) mouse brain infected with guinea pig pad virus, (b) mouse brain obtained from the twenty-fifth brain to brain passage in mice, (c) affected guinea pig pad or brain, and (d) monkey brain. The virus in guinea pig plantar tissue was least active, inducing the most protracted course of the experimental infection. The experimental disease initiated with this virus, however, was carried through at least seven brain to brain passages in *Macacus cynomolgus* monkeys and five in *rhesus* animals.

Both species of monkeys react similarly. The onset of signs of infection occurs, as a rule, from 4 to 7 days after inoculation. The first symptom observed is generalized weakness which progresses to definite paresis of the limbs, frequently associated with tremors and spasticity. Paralysis of one or more limbs and of the face supervenes occasionally in the later stages but at no time are there definite signs of meningeal involvement. Salivation was noted in 25 per cent of the animals and fever occurred on 1 or 2 days at the beginning of reaction. Death ensues on the 7th to 13th day after inoculation.

Filtrates of the brain tissue removed after death of the monkeys were found, on titration in mice, to contain virus in concentrations of 10^1 to 10^5. The cerebral tissue of each monkey, including those of the passage series, was also injected into the pads of guinea pigs, with the production of characteristic vesicular dermatitis. Similarly, brain tissue derived from the monkeys of the final serial passage, on transfer to the brain of mice and guinea pigs, induced characteristic experimental encephalitis. It is of interest that the plantar tissues of monkeys remain free from lesions after pad inoculation of the virus.

The microscopic lesions in the central nervous system and in the kidney and liver are comparable with those present in guinea pigs and mice, except that in monkeys there are more pronounced signs of productive inflammation; namely, invasion of perivascular sheaths and spaces with monocytes, localized areas of monocytic infiltrations in the gray and white matter of the brain and cord, multiplication of

glial nuclei and neuronophagia. Characteristic intranuclear inclusion bodies are readily found.

The observations just recorded show the neurotropic action of the vesicular stomatitis virus, now demonstrated in *rhesus* and *cynomolgus* monkeys. The course of events is not as rapid as in rodents. This may account for the more marked infiltrative lesions and for the more numerous inclusion bodies in the simians.

Effects on Other Animals

White and Hooded Rats.—These animals are susceptible to the action of the Indiana and New Jersey strains of vesicular stomatitis virus after intracerebral injection. Transmission of the experimental disease was effected through four serial brain to brain passages, the fifteen animals employed all succumbing to the disease within 7 to 10 days after inoculation. Introduction of the virus into the pads, however, leads to characteristic vesicular dermatitis of this tissue (9), as in the case of guinea pigs.

Young Chicks.—Chicks 24 to 48 hours old are unaffected after receiving intracerebrally from 0.05 to 0.12 cc. of virus filtrate. Eight different tests were made on sixteen birds.

Rabbits.—Rabbits are much more resistant to the virus, when injected in the brain, than are mice, rats, guinea pigs, or monkeys. Only three of fourteen test animals developed, within 7 to 9 days, signs of central nervous system involvement, terminating in complete flaccid paralysis of the posterior extremities. Pad inoculation is wholly without effect in the rabbit.

The employment by previous workers of the guinea pig as the experimental animal has led to the conception that the stomatitis virus is dermotropic in its action,—for of the dermal surface of the animal, only the plantar tissue has been proved to be uniformly susceptible to infection (3, 4, 9) From the results of the tests here recorded, we find that neurotropism, associated with a mild degree of viscerotropism, is a definite characteristic of the virus.

Recovery of Virus from Experimentally Infected Animals.—The employment of the white mouse as test animal, which is highly susceptible on intracerebral inoculation of the virus, has greatly facilitated its recovery. By mouse tests we have been able to demonstrate virus during the course of infection in the blood of the heart and peripheral circulation; in the submaxillary and parotid glands; in the brain, cord, and spinal fluid, and in the lung, spleen, liver, and kidney of mice, guinea pigs, and monkeys. In the monkeys it was

determined that the virus was present in the blood during the period from 24 hours after intracerebral inoculation, when the first test was made, to the onset of fever.

Cultivation of Vesicular Stomatitis Virus in Tissue Cultures.—The method of tissue culture in a medium consisting simply of minced chick embryos and Tyrode's solution has already been described (1 *b*). Two series of cultivation tests have now been performed, Series A with the Indiana strain and Series B with the New Jersey strain.

In Series A, filtrates of 1:10 suspensions of infected guinea pig pads or mouse brain pathogenic for mice in a dilution of 10^4 were used to initiate the cultures. At the present time this culture is in its thirty-fifth generation and is active in mice in a dilution of 10^5. The titer of the virus increased in this series to 10^{36}.

In Series B, the filtrate serving to initiate the tissue cultures was active in mice in a dilution of 10^3. The 58th generation now at hand was characteristically infective for mice in a dilution of 10^6, and the virus increased in titer to 10^{61}.

From these results it is evident that the virus can be cultivated in a medium consisting solely of chicken embryonic tissue suspended in Tyrode's solution. Other investigators (10) have succeeded in cultivating various viruses by this method. The fact is of interest that the virus of vesicular stomatitis was propagated with cells of the chicken, a species normally resistant to it.[3] Vesicular stomatitis virus has a generic relationship to the incitant of foot-and-mouth disease (9, 11); and it may be possible to cultivate the latter with equal ease.

VIRUS OF EQUINE ENCEPHALOMYELITIS

Meyer and his associates (5, 12) have stated that the filtrable,[4] glycerol-resistant encephalomyelitis virus is distinct from the incitant of botulism, "forage poisoning," Borna's disease, poliomyelitis, and apparently different from that of enzootic encephalitis of the Moussu-

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[3] It is of interest that the undifferentiated cells of chick embryos, especially of the membranes, are more susceptible to certain diseases than are the differentiated cells of the hatched chicks themselves. For a discussion and references see Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1932, **55**, 911; and Mackenzie, R. D., *J. Path. and Bact.*, 1933, **37**, 75.

[4] It was found that filtrability through Seitz' discs of the viruses of encephalomyelitis and of vesicular stomatitis is of the same degree; both pass the filters in concentration of 10^5, as determined by mouse inoculation tests.

Marchand type. We have confirmed their studies and elaborated the findings of the prior investigators. In our experience the virus has retained its infectivity in dilutions up to 1:10 million (instead of 1:10,000) and the white mouse has proved the experimental animal of choice instead of the guinea pig.

Effects on White Mice

These animals react uniformly when filtered or unfiltered, centrifuged, bacteria-free suspensions of the virus are introduced into the brain or nasal passages. The suspensions employed were prepared in the same manner as the vesicular stomatitis material. They consisted of infected mouse brain tissue, active when filtered in dilutions as high as 10^5, and when unfiltered as high as 10^7; and filtrates of affected guinea pig pad, and tissue culture material, infective up to 10^6 dilution. The experimental disease induced by means of nasal instillation of the virus was transferred by brain to brain passages through at least eight successive series, each of four to eight mice.

The fatal infection produced by the virus is characterized by the same clinical reaction and microscopic changes in the nervous system, liver, and kidney as are found in experimental stomatitis disease. The typical inclusion bodies are also detected in mice receiving the encephalomyelitis incitant intranasally, but not in animals injected intracranially. Moreover, precisely as in the case of experimental vesicular stomatitis, adult mice are refractory to intraperitoneal inoculation of the virus, but not infant ones. Another similarity consists in the shortening of the course of the experimental infection after twenty consecutive passages, from the usual 3 to 5 days to 2 days—transfers having been made from brain to brain every 48 hours.

As with the stomatitis virus, the incitant of encephalomyelitis produces no local dermatitis after its injection in the plantar tissues.

Effects on Guinea Pigs

The introduction of the virus into the brain of guinea pigs results in an infection indistinguishable from that of experimental vesicular stomatitis.

Serial pad passages at intervals of 48 hours were carried out in two sets of guinea pigs, with seventeen consecutive passages in the first and twenty-six in the second. This series of transfers was initiated with pooled, glycerolated guinea pig brain virus. As with the stomatitis virus, the encephalomyelitis virus present in the pads of the different passages induced fatal encephalitis after subdural inoculation of mice and guinea pigs.

The virus introduced into the plantar skin of guinea pigs shows vesicular reactions varying in degree and the serous exudate within the vesicles may be either blood-tinged or clear. While the plantar lesions induced by vesicular stomatitis are characterized, as a rule, by clear vesicular contents, it is known that strains

of this virus produce sometimes blood-tinged exudate. Hence there is no definite distinction between the two viruses in respect to the character of the vesicular fluid. The histopathological changes in the affected pads are, moreover, identical in the case of both incitants and the epithelial cells show the same type of intra-nuclear inclusion bodies.

As with the stomatitis virus, only the skin of the pad of the guinea pig shows dermatitis after inoculation with the virus of equine encephalomyelitis; and after five or six serial pad passages, the animals fail to exhibit signs of nervous involvement.

Effects on Other Animals

The reaction of monkeys, rabbits, and white rats to the virus after its introduction into the brain or pads is identical with that of the virus of vesicular stomatitis. Similarly, very young chicks are unaffected by intracerebral inoculation of the virus.

Establishment of Dermotropism.—We have already mentioned that the neurotropic incitant of encephalomyelitis, after five or six guinea pig pad passages, loses its property of affecting the central nervous system following pad injection of guinea pigs and then acquires dermotropism. An experiment was undertaken in which monkeys, rabbits, white mice, and white rats were inoculated into the plantar tissues with the modified, dermotropic virus. These animals, like the guinea pigs, also failed to show nervous disturbances. Hence it is evident that just as the dermotropic stomatitis virus can exhibit neurotropism, so can the neurotropic encephalomyelitis virus act as a dermotropic agent.

Recovery of Virus from Experimentally Infected Animals.—The virus can be recovered under similar conditions and from the same kind of tissues as in experimental stomatitis infections.

Tissue Cultures.—The virus can be cultivated in minced chicken embryonic tissue suspended in Tyrode's solution, in the same way as the stomatitis incitant. Fifty-three generations have been obtained to the present time; in the forty-ninth generation a dilution of the culture of 10^5 was capable of inducing fatal encephalitis in white mice, and the virus increased 10^{51} times.

IMMUNOLOGICAL REACTIONS OF THE VIRUSES

Methods.—Immunity tests were made by (a) introducing the virus into animals recovered from its effects and (b) serum neutralization. The mode of procedure in tests of the latter sort follows.

A 20 per cent suspension of virus-containing tissue in hormone broth of pH 7.5, was centrifuged at moderate speed for 10 minutes so as to clear the fluid of the grosser particles. 1 cc. of the supernatant fluid was diluted with 25 cc. of broth. Equal volumes of the diluted suspension and of the serum collected from recovered animals were mixed, placed in a 37.5°C. water bath for 2 hours, and then in an ice chest overnight. The mixtures were examined for the presence of virus by intracerebral injection into animals. In addition, neutralizing rabbit antiserum, effective against the strain used, was obtained by three successive subcutaneous injections at 5 day intervals, of 3 or 4 cc. of filtrate of guinea pig pad or brain tissues containing living virus. Ample controls were provided for each experiment.

Homologous Reactions of Vesicular Stomatitis Virus. (13).—The Indiana and the New Jersey strains were found to be immunologically distinct by both the *in vivo* and the *in vitro* procedure.

Guinea pigs recovered from the effects of plantar, subcutaneous, or intracranial inoculation of tissue cultures, guinea pig pad virus, or of brain material derived from infected mice, rats, guinea pigs, or monkeys, were resistant to later pad or intracerebral injection of the homologous strain of virus, irrespective of its source as to tissue or species of animal. The rabbit antiserum, or serum secured from recovered animals, showed corresponding homologous neutralization of the virus.

Homologous Reactions of Equine Encephalomyelitis Virus.—Guinea pigs recovered from the effects of this virus were shown to be immune to a later injection of the same virus. Rabbit antiserum, prepared with encephalomyelitis material, and the serum from animals recovered from the experimental disease, also inactivated the encephalomyelitis incitant. The methods employed in all these tests were precisely similar to those in the experiments with the virus of vesicular stomatitis.

Cross-Immunity Reactions.—On repeated trials cross-immunity reactions have not been found to occur between encephalomyelitis and stomatitis viruses.

SUMMARY

We have studied certain properties, additional to those previously described (3), of the virus of vesicular stomatitis of horses, and of the characteristic biological reactions of the virus of equine encephalomyelitis.

It has been found that the virus of stomatitis, ordinarily dermotropic, can acquire neurotropism and the neurotropic encephalo-

myelitis virus can, in turn, be rendered dermotropic in its action. The neurotropism in both instances is associated with definite, although not pronounced, viscerotropism.

Both viruses can bring about a similar infection in the white mouse, rat, guinea pig, rabbit, and *rhesus* or *cynomolgus* monkeys. Of these animals, rabbits show the lowest degree of susceptibility and mice the highest, especially after intracerebral inoculation. The mouse is the best animal for work with these viruses because of the uniform and rapidly lethal encephalitis which can be induced in it. Moreover, the mouse is highly sensitive to the instillation of the viruses in the nasal passages: 1 to 10 million dilution sufficing to induce a fatal encephalitis. The uninjured nasal mucosa of mice appears, therefore, to be as susceptible to experimental infection as the traumatized brain or pads of animals.

The microscopic changes accompanying the reactions to both viruses reveal, in rapidly lethal infections, pronounced destructive lesions in the cells of the central nervous system. When the experimental disease is more protracted in its course, however, these lesions are associated with beginning productive, inflammatory reactions, consisting chiefly of mononuclear infiltrations. In the latter instances, characteristic, intranuclear inclusion bodies can be more readily observed.

Both viruses can be cultivated with facility in the medium of minced chicken embryonic tissue suspended in Tyrode's solution, although 24 to 48 hour old chicks are refractory to artificial infection.

No cross-immunity reactions occur between the two strains of stomatitis virus or between them and the encephalomyelitis strain.

The viruses are evidently similar in many biological properties. In view of the fact that the horse is the natural host for both, it is suggested that they may be generically related. They are not, of course, identical since cross-immunity between them does not exist. The absence of cross-immunity does not, however, exclude the possibility of a generic relationship, for there are at least three immunologically distinct types of foot-and-mouth disease, two of vesicular stomatitis, and two of equine encephalomyelitis (14) virus.

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THE PULMONARY ARTERIAL PRESSURE IN NORMAL ALBINO RATS AND THE EFFECT THEREON OF EPINEPHRINE

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In a recent publication (1) we described lesions in the pulmonary arterioles of rats which had lived for some time in compressed air. These lesions indicated the possibility of pulmonary hypertension. Accordingly, it was decided to make direct determinations of the blood pressure in the pulmonary artery of rats after varying periods of exposure to the conditions prevailing in previous experiments (1, 2). Since, so far as we can learn, there have been no observations on the pulmonary arterial pressure in rats, it was necessary to determine the average pressure in a series of normal animals.

Methods
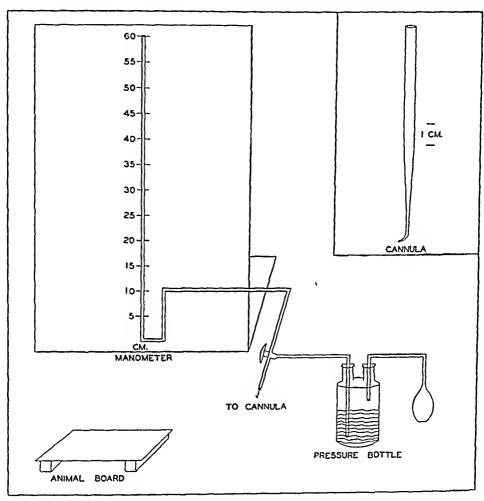
Albino rats from a standard colony were used. A total of thirty-four satisfactory determinations of the pulmonary arterial pressure was obtained. Some 70 per cent of the attempted readings proved successful after a suitable technique had been developed. The age of the majority of rats was between 4 and 5 months.

The pressures were determined directly on a water manometer (Text-fig. 1) by cannulating the pulmonary artery in a manner differing only slightly from the method described by Drinker and Went (3) for guinea pigs. The anatomical structure of the pulmonary arch in rats was such as to prevent the anchoring of a cannula as practiced by them. For this reason a small cannula was drawn from pyrex tubing (Text-fig. 1). The tip was turned from the shaft at a slightly obtuse angle. The end was ground on an emory wheel to a sharp, beveled point. The circumference of the tip increased abruptly as it approached the shaft, so that when the tip had pierced the arterial wall the increased circumference served as a wedge in the wall and thus prevented bleeding. The shaft was made long enough to be easily handled, ranging from 3 to 4 inches in length.

The cannula communicated directly with the water manometer through a three-way stop-cock (Text-fig. 1), one lead of which was connected with a pressure

bottle containing a solution of methylene blue in normal saline. By means of a pressure bulb attached to this bottle the height of the column of saline could be readily adjusted until it was in approximate equilibrium with the pulmonary arterial pressure.

Preparation.—The rats were anesthetized by an intraperitoneal injection of a 0.5 per cent solution of nembutal (sodium-ethyl (1-methyl-butyl) barbiturate)

TEXT-FIG. 1. Diagrammatic sketch of experimental apparatus.

in normal saline. Rats weighing less than 200 gm. received 60 mg. per kilo of body weight, while rats over 200 gm. received 70 mg. per kilo. Satisfactory anesthesia was obtained in 10–15 minutes. The rat was then placed on its back on an animal board. A tracheal cannula was inserted and positive pressure artificial respiration with an electrical pump was started. After an appropriate adjustment had been determined in the early experiments, the stroke of the pump

was not altered. The external jugular vein was then exposed to facilitate injection of heparin later. The thorax was opened by a longitudinal incision through the middle of the sternum from the tip of the xyphoid to the base of the neck. The thoracic walls were held apart by a self-retaining retractor. The thymus was dissected upward from the base of the heart and held back by a small clamp. Very little bleeding occurred when the incision was properly made through the middle of the sternum. Any bleeding points were temporarily clamped with hemostats. 3 or 4 minutes were allowed for clots to form in severed vessels before heparin was injected. A 0.6 per cent solution of heparin in normal saline was then injected, the number of cubic centimeters being determined by multiplying the weight in kilos by the factor 5.7. The usual quantity ranged from 1–1.5 cc. This amount of heparin was calculated to prevent the clotting of a quantity of cat's blood equal to twice the rat's blood volume. The latter was determined by assuming 85 cc. of blood per kilo of body weight.

Cannulation.—The cannula was filled with a 0.6 per cent solution of heparin and then connected to the manometer circuit by flexible rubber tubing. The water column was then raised to a height of 240–250 mm. The cannula was grasped in the right hand as a pencil, while the heart was gently retracted downward by a moist cotton pledget held in the left hand. In this way the pulmonary arch was exposed for a distance of 2–3 mm. distal to the pulmonic valve and the cannula was gently inserted with the tip directed toward the heart. When carried out successfully, the procedure required only a few seconds. The artificial respiration was discontinued for 5–10 seconds during the cannulation in order to steady the heart. As soon as the artery was entered, blood spurted into the tip of the cannula. The stop-cock was then turned so that the water column communicated freely with the cannula. If the water level tended to change immediately, it was readjusted as indicated until essentially in equilibrium with the pressure. Readings were then recorded at intervals of 15 seconds or 1 minute. In the first experiments the cannula was stabilized in plasticine, but since it frequently slipped out of the artery during the transfer from hand to plasticine, this method was abandoned. It was found more satisfactory for the operator to continue to hold it in place by hand.

Manometer readings were usually recorded in longhand; however, during experiments with epinephrine, in which marked changes in pressure occurred suddenly, it was found necessary to record the readings by camera at 5 second intervals according to the method described by Field and Drinker (4).

The results of all experiments in which cannulation was successful are shown in Table I. The average pulmonary arterial pressure for this series of thirty-four rats is seen to be 256 mm. H_2O.

The reliability of recording the pressure for only a few minutes is demonstrated by Table II, in which are presented the average pressure readings of fifteen rats that were recorded for periods ranging from 6

TABLE I

Rat No.	Age	Sex	Duration of experiment	Pulmonary arterial pressure	
				Average of	
				1st 5 min.	Total duration
	days		min.	mm. H_2O	mm. H_2O
10-31	126	M	54	279	276
10-12	140	F	38	279	284
10-10	140	F	35	256	256
10-39	129	M	20	263	260
10-33	126	F	18	257	267
10-34	128	M	13	284	273
10-40	131	M	12	244	247
10-36	128	M	10	250	249
10-51	334	F	10	303	309
10-41	130	F	9	284	261
10-54	155	M	8	253	255
10-60	159	M	8	276	276
10-76	125	F	8	229	225
10-32	126	F	7	228	223
10-19	143	F	6	277	278
10-45	133	M	6	259	258
10-17	141	F	5	267	267
10-53	154	M	5	272	272
10-58	157	M	5	312	312
10-61	159	M	5	266	266
10-66	—	—	5	265	265
10-69	125	F	5	255	255
10-70	125	M	5	299	299
10-71	125	M	5	201	201
10-72	125	M	5	187	187
10-73	125	F	5	183	183
10-74	125	F	5	220	220
10-23	133	M	4	—	281
10-35	128	M	4	—	219
10-52	334	F	4	—	243
10-59	159	M	3	—	239
10-62	159	M	3	—	275
10-65	305	M	3	—	233
10-63	303	M	2	—	291
Corrected average of series (34 rats)...............				257	256

to 54 minutes. The pressures on the 1st minute and for the first 5 minutes after the water column was in equilibrium as well as for the

total duration are shown. The difference between these readings is insignificant. The average pressure during the first 5 minutes after reaching the equilibrium was accepted as the standard in later experiments, because one was definitely able to ascertain that a constant level had been reached by then while further prolongation was unnecessary.

Accessory Factors.—The fact that sex, age, weight, and duration of anesthesia within reasonable limits bear no relationship to the pulmonary pressure is illustrated by the graphs in Text-fig. 2. The time
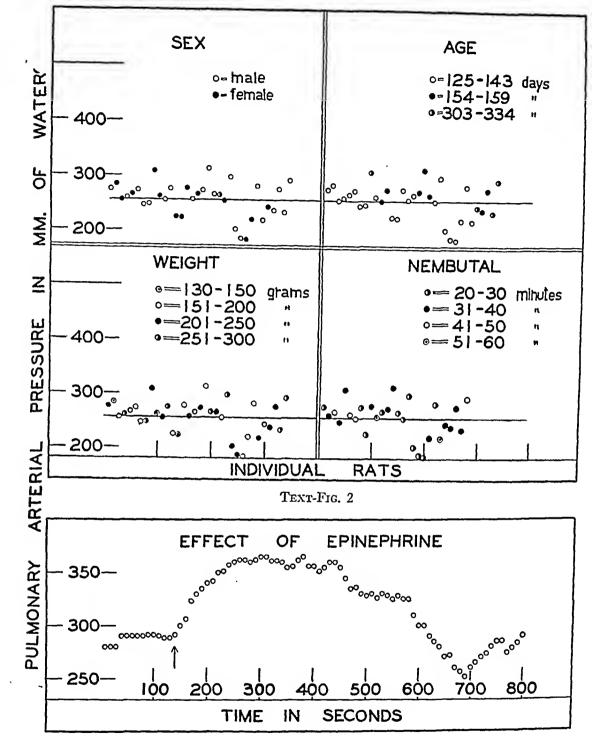
TABLE II

| Rat No. | Duration of experiment | Pulmonary arterial pressure | | |
| | | Average of | | |
		1st min.	1st 5 min.	Total duration
	min.	*mm. H₂O*	*mm. H₂O*	*mm. H₂O*
10-31	54	270	279	276
10-12	38	281	279	284
10-10	35	268	256	256
10-39	20	290	263	260
10-33	18	258	257	267
10-34	13	280	284	273
10-40	12	242	244	247
10-36	10	259	250	249
10-51	10	280	303	309
10-41	9	289	284	261
10-54	8	270	253	255
10-76	8	238	229	225
10-32	7	235	228	223
10-19	6	275	277	278
10-45	6	272	259	258
Average (15 rats).............		267	263	261

in the anesthesia graph represents the number of minutes elapsing between the injection of nembutal and the cannulation of the pulmonary artery.

The Effect of Epinephrine

The effect of epinephrine on the pulmonary pressure was studied in nine rats. An approximate 1:100,000 solution of epinephrine was used. It was administered intravenously in doses of 0.1–0.5 cc. The femoral and external jugular veins were used for injections.

TEXT-FIG. 2

TEXT-FIG. 3

TEXT-FIG. 2. These graphs illustrate the fact that the age, weight, and sex of the experimental animals, as well as the duration of anesthesia employed in this study bear no relationship to the pulmonary arterial pressure.

TEXT-FIG. 3. This graph shows the effect of epinephrine on the pulmonary arterial pressure in a representative experiment.

Epinephrine invariably caused an abrupt increased activity of the heart and a simultaneous elevation of the pulmonary pressure, the degree varying directly with the amount administered. A maximum pressure was soon reached but persisted for only a short time, gradually returning to normal. In the illustrated case 0.3 cc. of a 1:100,000 solution of epinephrine was injected into the femoral vein as indicated by the arrow (Text-fig. 3).

DISCUSSION

Three possible causes of error in making these determinations became apparent; namely, asphyxia, obstruction of the pulmonary circulation, and tension on the trachea. The first of these, when present, was usually caused by mucus or less frequently by a kink in the trachea at the junction with the cannula. Obstruction to the pulmonary circulation was due to faulty cannulation, resulting in either undue tension on the pulmonary arch or a disturbance of the normal relationships of the heart and mediastinal contents. Abnormal tension on the trachea probably caused its effect by altering the mediastinal relationships with impairment of the pulmonary circulation at the root of the lungs. All three of these accidents resulted in an abnormal temporary elevation of the pulmonary pressure to varying heights, followed by a sudden failure of the right side of the heart with a rapid fall in pressure ending in death unless remedied. After a little practice these difficulties were readily avoided. Early in the study it was realized that a cannulation was either immediately satisfactory in all respects or else it should be considered a failure and discontinued. When properly performed, no additional manipulation was necessary other than the adjustment of the water manometer as indicated. It was desirable to set the manometer a centimeter or two below rather than above the expected pressure. In this way a small quantity of blood was allowed to rise in the tip of the cannula. Cardiac pulsations could be followed here to ensure a patent cannula, while at times they were rather weak in the manometer.

The systemic blood pressure of rats under ether anesthesia has been determined in a series of 40 adults by Durant (5). The arithmetical mean in this group was 119 mm. Hg (1618 mm. H_2O). The ratio of the average pulmonary pressure in our experiments to the average

systemic pressure of 113 mm. Hg (6) is 1:6 which is identical to the ratio 1:6 given by Fuhner and Starling (7) for dogs. A comparison of the average pulmonary pressure found in this study to the average systemic pressure of 119 mm. Hg reported by Durant in a larger series of rats gives a ratio of 1:6.3 which is in close accord with the above ratios.

CONCLUSIONS

1. A satisfactory method for the direct determination of the pulmonary arterial pressure in rats is described.

2. The arithmetical mean of the blood pressure in the pulmonary artery in a series of thirty-four normal albino rats under nembutal anesthesia is 256 mm. H_2O (18.8 mm. Hg).

3. Intravenous epinephrine causes an abrupt but briefly sustained rise in the pulmonary arterial pressure with a gradual return to normal.

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PULMONARY HYPERTENSION IN RATS LIVING UNDER COMPRESSED AIR CONDITIONS

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PLATES 16 TO 18

In a previous publication (1) we described vascular lesions in the pulmonary arteries of rats following prolonged exposure to an environment of compressed air, having an oxygen tension of approximately 635 mm. Hg. The changes from normal in the pulmonary arterioles consisted of thickening and hyalinization of the walls with narrowing of the lumina. We commented upon the similarity of these lesions to those seen in the arterioles of the human kidney of patients with progressive vascular nephritis and hypertension.

The present study was undertaken in order to determine whether or not such vascular changes are accompanied by an increase in the pulmonary arterial pressure and to see if blood vessels other than the pulmonary arteries are affected.

Methods

1. Animals.—58 albino rats between the ages of 120 and 175 days were studied. Male and female rats were used in equal numbers. They were maintained on a standard diet which had proved satisfactory in other experiments (2).

2. Apparatus.—An environment of compressed air, containing a high oxygen tension, was obtained by using the compressed air equipment described by Thompson, Yaglou, and Van Woert (3). The humidity, temperature, and barometric pressure were kept constant by automatic regulators.

3. Conditions.—The barometric pressure was maintained at approximately 3040 mm. Hg (45 pounds gauge pressure) except for a drop to 2280 mm. Hg (30 pounds gauge pressure) for 10–15 minutes twice a week when the animals were being fed. Once a week the pressure was lowered to 2280 mm. Hg for 2–2½ hours while the pulmonary arterial pressure of a series of rats was being determined inside the pressure chamber. The oxygen tension, except for these insignificant periods, was approximately 635 mm. Hg, corresponding to an 83.6 per cent mixture

of oxygen at normal barometric pressure. The dry bulb temperature remained at 28°C. plus or minus 1°C., while the relative humidity ranged from 49–50 per cent. The total period of time during which the rats were kept in an environment of high oxygen tension identical with that of the previous experiments (1, 2) was 38 days.

4. *Determination of the Pulmonary Arterial Pressure.*—The pulmonary arterial pressure was determined on a series of rats after 3, 10, 17, 24, 31, and 38 days of exposure. It was measured directly on a water manometer connected with a cannula which was inserted into the arch of the pulmonary artery. A description of the method employed has been given in a previous paper (4).

5. *Pathological Technique.*—Each rat upon which the pulmonary arterial pressure had been satisfactorily determined was autopsied as soon as decompression was completed, usually within a period of 2–4 hours after death. The esophagus, trachea, heart, lungs, and thoracic aorta were removed in one piece, examined, and placed in fixative. After hardening, blocks of tissue were selected for microscopic study from similar areas in each organ. The brain, spleen, and liver were removed separately while the kidneys with the abdominal aorta and inferior vena cava were removed in one piece so as to enable us to obtain sections of the large abdominal vessels. Cross-sections of the femoral vessels and surrounding muscles were taken. Tissues were routinely fixed in Zenker's fluid. However, in a small series of rats, representative of varying periods of exposure to compressed air, the tissues were fixed in 10 per cent formaldehyde solution in order that fat stains on frozen sections might be made and thus the presence or absence of fat in sclerosed arteries be determined. Microscopic sections were stained routinely with methylene blue and eosin and phosphotungstic acid hematoxylin. In representative groups of rats, frozen sections from all organs were stained with Scharlach R.

The Pulmonary Hypertension

In a preliminary study of normal rats (4) the average pulmonary arterial pressure in a series of 34 animals was found to be 256 mm. H_2O. The results of all determinations in the present investigation are presented in Table I.

The pulmonary arterial pressures were within normal limits during the first 17 days of exposure except for one unusually low reading after 3 days exposure, when many of the animals showed the effects of acute oxygen poisoning, and one high reading on the 10th day. Definite pulmonary hypertension was present in one rat after 24 days of exposure while all of the other readings made on that day corresponded to the upper limits of the normal series.

All animals subjected to the environment of compressed air for 31 and 38 days respectively were found to have definite pulmonary hypertension.

TABLE I
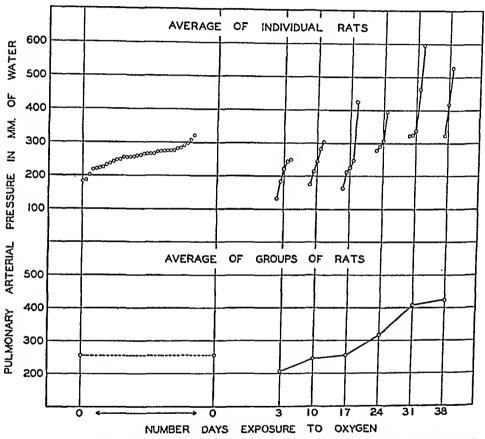
Pulmonary Arterial Pressure in a Series of Rats Exposed to Compressed Air for Varying Periods of Time

Animal No.	Age	Sex	Duration of experiment	Duration of exposure to 83 per cent oxygen tension	Pulmonary arterial pressure
	days		min.	days	mm. H_2O
11-1	139–140	M	$3\frac{1}{2}$	3	245
11-4	139–140	M	3	3	133
11-5	122–140	F	5	3	250
11-7	122–140	F	7	3	184
11-8	122–140	F	5	3	222
				Average....207	
11-9	146–147	M	5	10	176
11-10	146–147	M	4	10	214
11-11	129–147	F	5	10	304
11-12	146–147	M	5	10	244
11-13	129–147	F	5	10	283
				Average....244	
11-14	136–154	F	5	17	212
11-16	153–154	M	5	17	249
11-17	136–154	F	6	17	227
11-18	136–154	F	5	17	425
11-19	153–154	M	5	17	163
				Average....255	
11-21	160–161	M	5	24	309
11-22	160–161	M	5	24	394
11-25	143–161	F	2	24	279
11-26	143–161	F	$1\frac{1}{2}$	24	290
				Average....318	
11-28	167–168	M	4	31	463
11-29	167–168	M	10	31	339
11-30	150–168	F	5	31	322
11-31	150–168	F	5	31	597
11-33	167–168	M	3	31	324
				Average....409	
11-42	174–175	M	$1\frac{1}{4}$	38	419
11-44	157–175	F	5	38	322
11-45	174–175	M	5	38	529
				Average....423	

The average pulmonary arterial pressure of the normal and experimental series of rats are plotted in Text-fig. 1, both as individual rats and as groups, showing the rapid elevation of pressure that occurs in weekly intervals after the 24th day of exposure.

No attempt was made to determine the systemic arterial blood pressure of rats during their stay in an environment of compressed air.

TEXT-FIG. 1. The pulmonary arterial pressure in normal and experimental animals plotted individually and as groups, illustrating the gradual development of pulmonary hypertension during exposure to compressed air.

However, such determinations were made on a series of five rats which had been exposed for 38 days, decompressed, and kept at normal barometric pressure for 7–10 days. In these animals the carotid artery was cannulated and the arterial blood pressure measured directly on a mercury manometer. After the initial carotid arterial pressure had

been recorded, the pulmonary artery was cannulated as in previous rats, in an effort to simultaneously measure the pulmonary arterial pressure.

The average carotid arterial blood pressure in these five rats was 55 mm. Hg, the lowest individual average pressure being 48 mm. Hg, and the highest 59 mm. Hg. Thus in each instance the systemic blood pressure of the exposed rats was slightly less than one-half of the average normal (113 mm. Hg) in four rats which we examined and the average normal systemic pressure of 119 mm. Hg as reported by Durant (5). Although the number of rats upon which these determinations were made was small, the consistently low pressure readings in each instance provide additional evidence of an important degree of arterial obstruction in the lesser circulation. Gibbon, Hopkinson, and Churchill (6) demonstrated that when the pulmonary artery in cats was obstructed to from 60 per cent to 85 per cent of its cross-sectional area, there was a reduction in cardiac output, attended by a fall in the systemic blood pressure and a rise in venous pressure.

An attempt to make simultaneous determinations of the systemic and pulmonary arterial pressures failed in two of the five rats. In the remaining three rats the average pulmonary arterial pressure was 296 mm. H_2O which was considerably higher than the average pressure of 179 mm. H_2O in three control rats in which simultaneous pressure readings were made. The fact that the average pulmonary pressure in both the exposed and normal rats is lower than in corresponding groups of animals in which only the pulmonary artery was cannulated, suggests that the added manipulation and blood loss entailed in measuring the systemic pressure may have resulted in readings that were not as high as the actual pulmonary pressure in these animals.

Pathological Findings

Gross and microscopic examination of the brain, liver, and kidneys did not show any constant abnormality. Microscopic examination of the blood vessels of these organs as well as of the aorta in both the thoracic and abdominal portions and the femoral arteries, revealed no pathological change. The spleen in all animals appeared normal on gross examination but in practically all instances there was a recognizable increase in the amount of hemosiderin in the exposed rats. Scharlach R stains made on frozen sections failed to demonstrate the presence of pathological fat in the blood vessels or parenchyma of any of the above organs.

The heart and lungs alone showed constant or important changes from normal.

Macroscopic examination of the heart after death did not reveal as striking changes as did direct observation of the exposed and functioning heart while the pulmonary pressure determinations were being made. At that time, in rats exposed to compressed air for 24 days or more, the heart action usually appeared more forceful than normal and the right ventricle was constantly dilated so that its prominent borders partially obscured the adjacent margins of the left ventricle. The conus arteriosus instead of tapering gradually into the pulmonary artery as it did in normal rats was markedly ballooned out and obscured the ventriculoarterial junction.

In one instance (Rat 11-28) very interesting correlations between the markedly increased pulmonary pressure and the pathological changes in the right ventricle were possible. In this rat the right ventricle was tremendously dilated when first exposed. When the cannula was inserted into the pulmonary artery, the pressure rapidly mounted to 500 mm. H_2O, where it remained in equilibrium for a few seconds and then rapidly fell as the right ventricle became even more dilated, slowed down, and almost ceased beating. By lowering the pressure in the manometer to a subnormal level, enough blood was expelled from the right ventricle to allow it to recover temporarily and the elevated initial pressure was almost totally regained and sustained for $2\frac{1}{4}$ minutes. At this time the right ventricle again became decompensated and failed to revive. Examination of the heart after death showed an area 4 x 3 mm. in diameter in the wall of the right ventricle which had the gross characteristics of a cardiac aneurysm. This portion of the myocardium bulged out from the surrounding musculature and when sectioned across was found to consist of a very thin fibrous membrane. Microscopic examination of this lesion showed that the muscle fibers had almost entirely degenerated, leaving only thin dense scar tissue which presumably had become markedly stretched out. Two other hearts showed similar but smaller defects in the right ventricular musculature which were recognizable on macroscopic examination. These hearts were the most damaged of any in the series, although microscopic examination revealed slight to moderate scarring in the right ventricle of most of the animals which had been exposed to compressed air for 31 days or longer. Such areas of scar formation were usually small and consisted only of connective tissue increase between muscle fibers. In a few instances, however, there was a slight infiltration with mononuclear leucocytes and, in one example, on the sixth day of exposure, a widespread and active myocarditis of the right ventricle was present. In this instance the muscle fibers were degenerating, as shown by large amounts of finely divided particles of fat and there was a heavy inflammatory cell infiltration. In a number of sections the auricular musculature showed similar areas of inflammatory cell infiltration or connective tissue increase between muscle fibers.

Macroscopic and microscopic examination of the lungs confirmed in most respects our previous observations (1). On the 3rd day of exposure the lungs showed an acute inflammatory reaction characterized chiefly by marked perivascular edema, alveolar edema, and pleural effusion. In numerous areas the alveoli

contained fibrin and a scattering of mononuclear and polynuclear inflammatory cells. The alveolar walls showed an increase in polymorphonuclear leucocytes. As in the previous series of rats, the trachea, bronchi, and bronchioles showed no evidence of injury except in occasional instances in which a small amount of muco-purulent exudate was present within the larger bronchi. In the present study it was noted that the pulmonary arterioles stood out more prominently than normal. This fact was due chiefly to the surrounding zone of edema and dilatation of the accompanying lymphatics. However, because of a slight alteration in the stain-ing quality of the media and because of a tendency to separation of fibers in the arterial walls in occasional vessels, we believe that even at this early period there was injury to the pulmonary arteries (Fig. 1). One of the most striking histological abnormalities was the apparent increase in the thickness of the walls of occasional arterioles with a corresponding narrowing of the lumina (Fig. 2) which in occa-sional vessels gave the appearance of complete closure. The large pulmonary arteries were not appreciably thickened after an exposure of 3 days. There was, however, marked perivascular edema about them, and slight to moderate fraying of the fibers in the adventitia. The fibrils of the connective tissue about many of the large pulmonary veins were spread apart by edema. This abnormality was not as extensive or as constant as in the case of the pulmonary arteries. In several sections the walls of the large veins showed scattered areas of degeneration characterized by edema, disappearance of muscle fibers, and a moderate to heavy infiltration of inflammatory cells which consisted chiefly of mononuclear leucocytes. Scattered polymorphonuclear leucocytes were also present. Such lesions were not accompanied by thrombus formation.

After more prolonged exposure the alterations noted in the alveolar units of the lungs were essentially the same as those previously observed and described (1), the most striking change being hyperplasia and hypertrophy of the alveolar lining cells. There were also progressive changes in the large pulmonary arteries which became prominent after 10 days of exposure.

A tabulation of the measured thickness of the walls of the large pulmonary arteries and aorta of the individual rats throughout the experiment is given in Table II. Such measurements were made on microscopic sections with an ocular micrometer which had been cali-brated on a stage micrometer. While it is true that some variations in the diameters are no doubt due to variations in the locations at which the measurements were made and to the difference in the age of the various rats, nevertheless we are satisfied that the averages by periods of exposure are approximately correct. Sections from approximately the same anatomical levels were selected and only those arteries which were squarely cut across were measured. One sees from these meas-urements of the large vessels that the thickness of the wall of the

aorta remained practically constant, whereas the wall of the pulmonary artery doubled in thickness within a period of 10 days and became three times its normal thickness after 1 month. Increased resistance met in attempting to insert the cannula through the wall of the main pulmonary artery in all animals exposed to compressed air for 24 days or longer provided additional evidence of this sclerosing process. No variations from normal were noted on microscopic examination in any arteries of the systemic circulation.

TABLE II

A Comparison of the Average Thickness of the Large Pulmonary Artery and Aorta in Normal Rats and Rats Exposed to Compressed Air for Varying Periods of Time*

Animal series	Duration of exposure to compressed air	No. of animals in each group	Average thickness of aortic wall	Average thickness of wall of pulmonary artery
	days		*mm.*	*mm.*
Control	0	13	0.0982	0.0477
Experimental	3	5	0.1029	0.0531
"	10	5	0.0929	0.1029
"	17	5	0.1095	0.1162
"	24	3	0.0941	0.0941
"	31	5	0.1062	0.1382
"	38	3	0.1328	0.1992
"	38†	5	0.1162	0.1714

* The thickness of the blood vessel walls was measured with an ocular micrometer, calibrated on a stage micrometer.

† Animals decompressed after 38 days exposure and kept at normal atmospheric pressure for 7 to 10 days.

The microscopic changes noted in the large pulmonary arteries after 3 days of exposure consisted of a slight increase in prominence of the alternate layers of elastic tissue and smooth muscle with some fraying of the adventitia, presumably due to perivascular edema (Figs. 3 and 4). After 10 days of exposure the layers of muscle cells and elastic tissue appeared even more prominent and slightly thickened. At that time a progressive increase in connective tissue external to the media became evident. In the beginning this tissue was loose textured and slight in amount. With each succeeding week of exposure this fibrous tissue increased and became condensed so that in the late stages of the experiment (24 days or more) a thick layer of dense hyalinized fibrous tissue had formed (Fig. 5). As in the previous experiment, this occasionally resembled fibrocartilage. The

marked increase in the thickness of the arterial walls was due largely to this fibroblastic proliferation outside of the media and not to as extensive an alteration in the media as we had formerly thought.   It is also worthy of emphasis that the thickening process in the large pulmonary arteries began to make its appearance at an earlier date than we previously stated (1).   No comparable changes were detected in the walls of the large pulmonary veins in the late stages of the experiment.

Although a moderate increase in thickness of the walls of the small pulmonary arterioles, together with a visible increase in their number, was seen in this series of rats (Figs. 6–8) the marked degree of hyalinization described in the former series had not uniformly developed by the end of this experiment.   In the previous study (1) we commented that: "After 1 month of exposure, the small arterioles of the lungs became prominent and apparently more numerous. . . . Their walls were thickened and the lumina narrowed.   Later, hyalinization of the walls occurred and occasionally thrombosis. . . . These changes in the walls made the small vessels stand out prominently, which probably accounted for the apparent increase in number."   Owing to the fact that in this experiment exposure to compressed air was not carried beyond the 38th day, it seems reasonable to believe that the small arterial lesions were not uniformly as marked only because insufficient time had elapsed.

DISCUSSION

The anatomical and physiological alterations demonstrated in this investigation can be partially explained by a correlation with observations already established in clinical and experimental studies by other workers.

Of the mechanical factors stated by Wiggers (7) to conceivably alter the pressure and volumes of blood in the pulmonary vessels when operating separately or together, namely (1) the minute output of the right ventricle, (2) the resistance and capacity changes in the pulmonary circuit, (3) back pressure resistance produced in the left heart by changes in the systemic circuit, the second factor would seem to apply more directly in the present interpretation.

In these studies, pathological changes have been found in the lungs which are of such a nature that one might expect them to offer increased resistance to the pulmonary circulation.   During the stage of acute oxygen poisoning there is injury to the arterioles as evidenced by a thickening of the walls and narrowing of the lumina together with alterations in staining properties, as well as capillary injury which results in pulmonary edema and marked pleural effusion.   The latter

must necessarily cause considerable lung compression, and lung compression by pleural exudates has been cited as a probable cause of elevation of pulmonary arterial pressure (8). With continued exposure the pulmonary edema disappears but the arteriolar changes persist and become more marked and are accompanied by simultaneous hyperplasia and hypertrophy of the alveolar lining cells with resultant thickening of the alveolar walls and a decrease in the number of visible blood-filled capillaries. Patchy areas of atelectasis are constantly associated with these changes.

Other factors being equal, it would be expected that all of these changes, whatever the exciting cause, would increase the resistance to blood flow in the lesser circulation and consequently give rise to an elevation of blood pressure in the pulmonary artery and right ventricle beginning with the onset of acute oxygen poisoning. The fact that pulmonary hypertension was not demonstrable by the method employed (4) until after 24 days of exposure, does not necessarily mean that an elevation of pressure was not present in the intact rat prior to that time. During the period in which there was pleural effusion, the fluid escaped on opening the thorax to expose the pulmonary artery, thereby eliminating the effect of lung compression on the pulmonary resistance. Subsequent to this the increased resistance caused by atelectasis and early changes in the alveolar walls and arterioles is probably removed when the thorax is opened and the lungs subjected to forced artificial respiration, so that the existence of pulmonary hypertension cannot be demonstrated until after the 3rd week of exposure when the alterations in the arterioles become sufficiently advanced to cause permanent obstruction to the pulmonary circulation in spite of the minimizing influence of the experimental methods.

The increase in thickness of the walls of the large pulmonary arteries which was constantly present after the 10th day and progressive throughout the duration of the experiment was present before pulmonary hypertension was demonstrable. It would seem reasonable to believe that this change was due to prolonged stretching from overdistension secondary to pulmonary hypertension which we believe to be present from the beginning, although not detectable by this experimental method until later. This sclerosing process consisted chiefly of the formation of new connective tissue around the media of

the wall, thereby differing markedly from any common type of arteriosclerosis. While it is true that this fibrosis occurred in the regions where marked edema was present during the acute stages of oxygen poisoning, the fact that comparable fibrosis did not occur around veins where edema had also been demonstrated would seem to rule out tissue edema as the cause for this prominent anatomical abnormality. It appears improbable that such changes could have been a factor in the production of the pulmonary hypertension.

Dilatation and hypertrophy of the right side of the heart and dilatation of the conus arteriosus are common signs of pulmonary hypertension in man (9). These changes were observed in rats dying of acute oxygen poisoning and were likewise prominent in surviving rats on prolonged exposure. Increased venous pressure is given as one of the important physical signs of pulmonary hypertension in man (9) and decreased systemic blood pressure with an increase in venous pressure has been demonstrated in animals to result from constriction of the pulmonary artery (6). In the present series of animals a low systemic blood pressure was found to be present in each of a small series of rats after 38 days of exposure to compressed air. Thus the anatomical and physiological alterations observed in rats in this study resemble in many respects the outstanding features of pulmonary hypertension in the human subject.

SUMMARY

1. Pulmonary arterial hypertension was demonstrated in a series of rats that had been kept for 24–31 days in an environment of compressed air, having a barometric pressure of 3040 mm. Hg. The partial pressure of oxygen was 635 mm. Hg, which is equivalent to an 83.6 per cent oxygen mixture at normal barometric pressure.

2. Sclerosing changes in the pulmonary arterioles have been observed which precede the development of demonstrable hypertension in the pulmonary circulation. These vessels showed histological changes that were indicative of injury after 3 days of exposure. There was a thickening of the walls which stained more intensely with eosin, as well as marked perivascular edema and often a narrowing of the lumina. Progressive thickening, narrowing, and hyalinization of the pulmonary arterioles occurred later, after the disappearance of

perivascular edema. These changes appeared very similar to the renal arterial lesions seen at autopsy in patients dying from malignant hypertension.

3. Pathological examination did not reveal significant or constant changes from normal in any organs except the lungs and heart. The blood vessels of the systemic circulation showed no pathological change.

4. The walls of the large pulmonary arteries increased in thickness rapidly after the 3rd day of exposure. This change was due to the progressive formation and condensation of fibrous tissue outside of the media and to a lesser extent to thickening of the alternate layers of elastic tissue and smooth muscle in the arterial wall.

5. Marked dilatation of the right ventricle and conus arteriosus as well as small areas of scar tissue formation in the right ventricle were present on prolonged exposure. A few hearts showed larger areas of fibrosis that were visible on macroscopic examination.

6. The systemic arterial blood pressure of a small series of rats exposed to compressed air for 38 days and examined 7 to 10 days after decompression was in each instance less than one-half the average normal pressure.

7. The findings in this study are consistent with the clinical and pathological signs of pulmonary hypertension in man.

8. The anatomical alterations observed in the alveolar units of the lungs were essentially the same as those previously described (1).

9. A method has been devised whereby pulmonary arterial hypertension, accompanied by important sclerotic changes in the arteries of the pulmonary circulation can be induced for investigation.

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EXPLANATION OF PLATES

PLATE 16

FIG. 1. Lung of a rat exposed to compressed air for 3 days showing marked perivascular edema and leucocytic infiltration about large and small pulmonary arteries.   One should also note the intensity with which the small arterioles have stained and the marked thickening of the wall and narrowing of the lumen of one arteriole.   Section stained with eosin and methylene blue. $\times$ 82.

FIG. 2. A thickened and narrowed pulmonary arteriole, such as were occasionally seen after 3 days of exposure to compressed air.   Eosin and methylene blue. $\times$ 215.

FIG. 3. Large pulmonary artery in a normal rat.   Eosin and methylene blue. $\times$ 86.
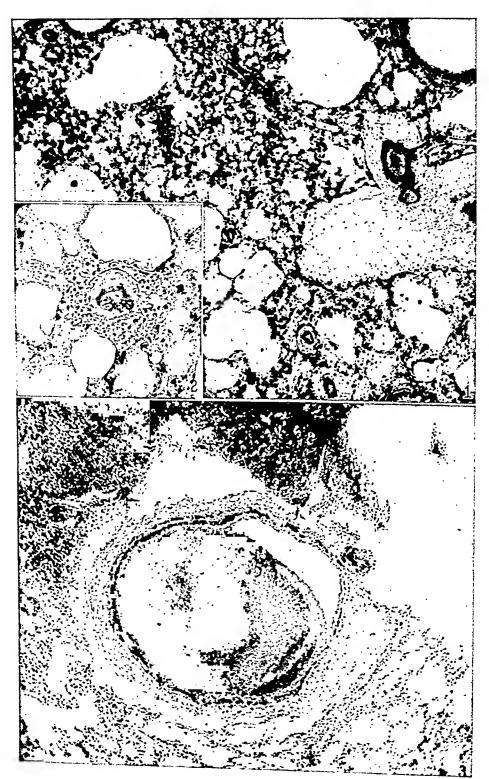
PLATE 17

FIG. 4. A large pulmonary artery of a rat exposed to compressed air for 3 days. Note the marked perivascular edema, cellular infiltration, and fraying of the adventitia.   Eosin and methylene blue. $\times$ 82.

FIG. 5. A large pulmonary artery comparable in size to Figs. 3 and 4, showing marked increase in fibrous tissue external to the media.   Rat exposed to compressed air for 38 days.   Hematoxylin and eosin. $\times$ 82.

PLATE 18
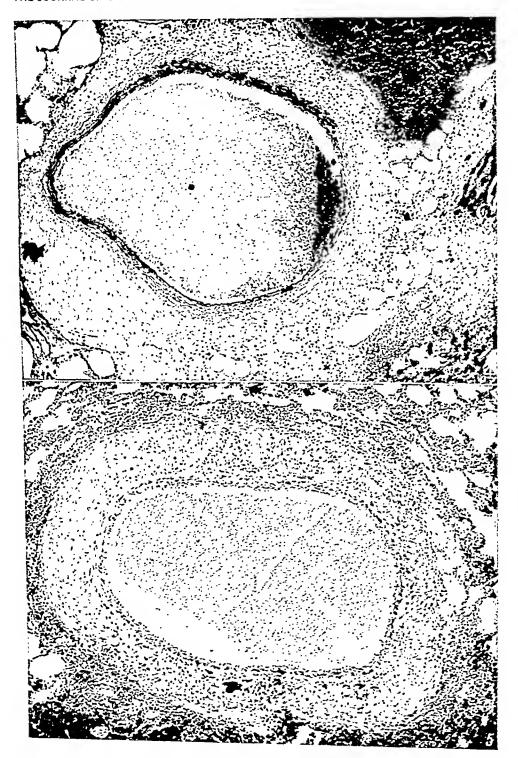
FIG. 6. Lung tissue of a rat which had been exposed to compressed air for a period of 38 days.   Note the increased cellularity of the alveolar walls and the numerous and prominent thick walled pulmonary arterioles.   Hematoxylin and eosin. $\times$ 82.

FIG. 7. Three thick walled hyalinized arterioles.   Rat exposed to compressed air for 38 days.   Hematoxylin and eosin. $\times$ 180.

FIG. 8. A greatly thickened, narrowed, and hyalinized pulmonary arteriole. Duration of exposure 38 days.   Hematoxylin and eosin. $\times$ 500.

PLATE 17

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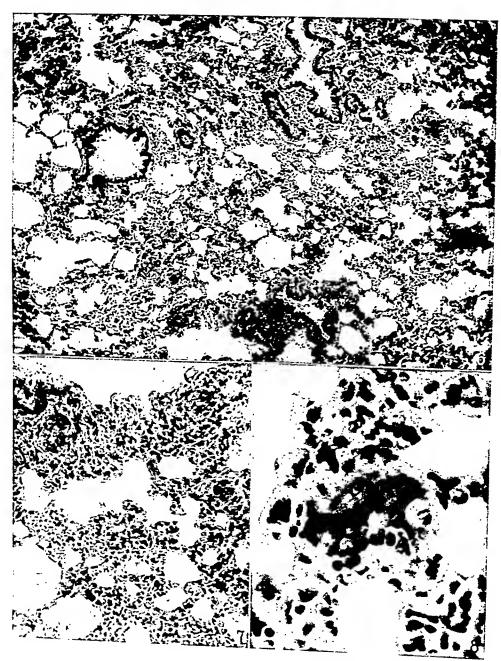
(Bennett and Smith: Pulmonary hypertension)

ENERGY METABOLISM OF THE FAILING HEART

By GEORGE DECHERD, M.D., AND MAURICE B. VISSCHER, M.D.

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Starling (1915) showed that the normal heart dilates only when increased work is thrown upon it. In disease the heart dilates without any increase in work imposed upon it. It is obviously of importance to know in what particulars the failing heart differs from the normal. The experiments recorded here amplify the results of Starling and Visscher (1927) in elucidating this problem. In their experiments it was found that the energy liberation in the normal heart is fixed by the size of the heart at the beginning of contraction; *i.e.*, at the end of diastole. The size of the heart is a measure of the length of the muscle fibers; consequently their results may be expressed by saying that the quantity of energy liberated in contraction is determined by the length of the fiber at the time of contraction.

As a matter of fact, we are more fundamentally interested in the amount of work the heart can do than we are in the total energy it can liberate. Therefore the proportion of the total energy which can be put to useful work, which is the efficiency, becomes a matter of first importance. In cardiac decompensation in man, as well as under certain conditions in experiments on hearts of lower animals, the heart dilates progressively in spite of a uniform or even decreasing load. One ordinarily speaks of such a heart as having poor tone. The heart of a decompensated patient does little work in spite of its enormous size and its apparent effort. The question as to whether the heart under these circumstances is defective in liberating less energy than a normal heart would, or is defective in being unable to use the energy for work in ejecting blood, was investigated by Starling and Visscher (1927). They found that when the mammalian heart lost its normal tonus, and dilated enormously in order to do amounts of work that when fresh it could do at small volume, the total energy

liberation was not at all diminished; only the proportion of the total energy which could be converted into work was diminished. That is to say, the efficiency of the heart muscle as a machine was found to fall as the condition of the heart deteriorated. There was no lessening in the quantity of energy made available.

It has been necessary to reinvestigate these problems because Stella (1931) questioned the reliability of the earlier results. Decherd and Visscher (1933), and Moldavsky and Visscher (1933, 1934), have indicated the errors which led Stella to fallacious conclusions. Certain observations upon failing hearts are described here because of their importance to an understanding of the physiology of decompensation.

In these experiments the total energy liberated in contraction was calculated from measurements of the oxygen consumption. The latter was measured by a modified Barcroft differential manometer method, observing the decrease in volume of oxygen in the vessels containing the turtle heart and the perfusion fluid, while the carbon dioxide was being removed from the air by potassium hydroxide present in a porous plate suspended in the vessel. A compensating vessel of equal air volume automatically corrected for temperature changes, which were within 0.005°C., since the bath itself was controlled within that limit. The relatively large volume of oxygen in the system made such accuracy in temperature control necessary. The energy converted to work was calculated from the pressure and the output. The pressure was measured with a membrane manometer and the output was observed by employing the closed chamber around the heart as a cardiometer, recording changes in the volume of air within it by means of a properly calibrated tambour. A more detailed description of the apparatus and methods used has been published by the authors (1933) in a previous paper.

A heart perfused with Ringer's solution for a long time spontaneously dilates and fails to eject as much blood as enters between contractions. It can be therefore said that when the heart begins to fail the condition becomes evident in an inability to keep up its output per beat at a given diastolic volume. In Fig. 1 are shown the results of a typical experiment in which the volume in diastole was kept constant while the heart was failing. More than twenty-five

concordant experiments have been performed. It can be seen that the work the heart was able to do falls off very rapidly. It is equally obvious that the energy liberated per beat does not diminish at all during the time of observation. This has been the invariable result of many observations. It appears, therefore, that in the decompensating heart the defect is not in a failure of the heart to liberate enough energy to do its work. It can still liberate as much energy as it ever could. Its defect lies rather in an inability to employ the energy for useful work. This is entirely aside from any valvular defects which,
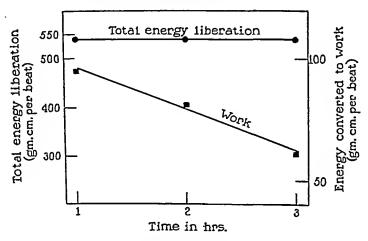
FIG. 1. In this experiment the ventricle was held at constant diastolic volume over several hours. The energy liberation remains constant, but the work done falls off markedly as the condition of the isolated heart deteriorates.

of course, also diminish the efficiency of the heart. The machine which does mechanical work with the energy of contraction is out of order. The extent of this defect is measured by the efficiency of the heart. In Fig. 2 are plotted the results of two experiments in which the efficiency of the failing heart was measured for several hours during which its condition was becoming steadily worse. When this impairment occurs the ventricles are obliged to dilate to a greater diastolic fiber length in order to do the work imposed upon them. Thus it is apparent that the dilated heart, which one describes as having poor tone, is in reality simply one with low mechanical efficiency.

These observations have many implications for clinical medicine. It is at once apparent that the failing heart is not in need of fuel, but rather of materials for repairs. Visscher and Müller (1927) and Bodo (1928) investigated certain agents which improved the condition of the isolated heart and found that insulin was conspicuous among all substances employed in restoring the tone or, in other words, efficiency, of isolated working hearts. Further experimentation along this line is in progress in this laboratory.
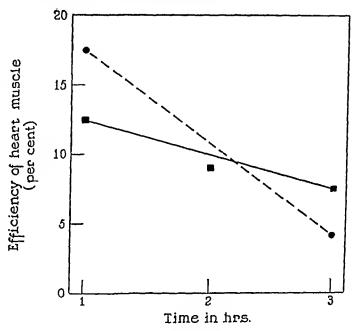
FIG. 2. The results of calculations of efficiency in two experiments are plotted in this figure. It will be noted that the mechanical efficiency gradually falls off over the period of several hours' observations. The diastolic volume was held constant throughout.

There are also agents which diminish the efficiency of heart muscle. Starling and Visscher found that adrenalin, although it caused a great increase in total energy liberation, produced a marked lowering of efficiency, and therefore left the heart in worse condition than it had been before. Although temporarily stimulating to energy liberation by the heart, adrenalin eventually leaves the heart muscle less efficient and must therefore frequently be very harmful to a weakened myocardium.

SUMMARY

The failing heart is found to liberate energy in contraction to the same extent at any fiber length as the normal heart muscle, but it is unable to convert as much of the energy into useful work. Its efficiency as a machine is impaired. In the light of these observations the word "tonus" as applied to heart muscle becomes synonymous with "efficiency." The implications of these findings in applied cardiology are discussed.

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SWINE INFLUENZA

V. Studies on Contagion

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In experiments published earlier (1–3) it has been shown that both a filtrable virus and the organism, *H. influenzae suis*, are etiologically essential to the production of influenza in swine. All five strains of the disease studied have been highly contagious. Animals infected by pen exposure to cases of the disease developed typical influenza identical with that produced by inoculation intranasally with virus and organism. Furthermore, in swine infected by contact both the virus and the organism could be demonstrated as having transferred.

During the spring and summer of 1931, when a strain of the disease was being passed through swine every 6 weeks to preserve it for subsequent experimental work, a change in its contagious character occurred. It is the purpose of this paper to describe and interpret the change.

A Change in the Contagious Character of a Strain of Swine Influenza

The change in contagion occurred in a strain of the disease in which the etiological components were Virus 15, obtained originally from Iowa in December, 1930, and *H. influenzae suis* Culture 451, the bacterial component of the first strain of swine influenza obtained from Iowa in November, 1928. This combination of virus and organism had been used in inducing influenza in swine during intensive work with the disease throughout the winter of 1930–31. During this time it regularly caused, when administered intranasally to swine, an influenza that was characteristic in all respects and that was known to be fully contagious as late as March, 1931.

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The general plan followed for preserving the virus during intervals between experiments was to store it in the refrigerator either in 50 per cent glycerol or dried by Swift's method (4). During the spring and summer of 1931, when the change in contagion to be discussed took place, the virus was stored only in the dried state. The bacterial component of the etiological complex, *H. influenzae suis*, which does not regularly survive such storage, was maintained by weekly transfer on plain agar slants containing 0.75–1 cc. of sterile defibrinated horse blood at their bases. The usual procedure at each 6 weeks passage was to prepare an approximately 5 per cent suspension of the dried virus and administer 10 cc. of this suspension mixed with 1 cc. of the bloody condensation fluid from a 24 to 48 hour culture of *H. influenzae suis* intranasally to each animal to be infected. Clinically and pathologically characteristic swine influenza developed in the swine inoculated at each passage. The animals were sacrificed on the 3rd or 4th day following infection and pathological lung, bronchial lymph nodes, and bronchial exudate were saved to dry by Swift's method to furnish virus for the next passage. Cultures of *H. influenzae suis* were not saved from the passage animals. A stock laboratory strain of the organism was used throughout in conjunction with the passaged virus.

In September, 1931, during the time that the disease was being maintained by passage at 6 week intervals, an attempt was made to pass it by contact. Instead of contracting swine influenza, as was to be expected from previous experience with the disease, the contact animal developed only a very mild, afebrile, and transient illness similar to that described in an earlier paper (3) and called the "filtrate disease" because it is produced by the intranasal infection of swine with the swine influenza virus alone.

In a series of seven subsequent contact experiments in which normal swine were exposed to animals suffering from the apparently typical influenza induced by Strain 15 swine influenza virus and *H. influenzae suis* Culture 451 no cases of influenza occurred. Instead, all of the exposed swine developed, after an incubation period of from 2 to 6 days, only a very mild and transient illness which clinically and pathologically resembled the filtrate disease. Bacteriological examination of their respiratory tracts failed to reveal the presence of *H. influenzae suis*, further supporting the view that we were dealing with the filtrate disease and not merely with an unusually mild form of swine influenza. The illness contracted by the animals infected by contact was shown to be transmissible in series by pen exposure, and two swine allowed to recover were found later to be solidly immune to swine influenza as

induced by intranasal inoculation with virus and *H. influenzae suis* (5). The above facts, considered collectively, were in accord with the view that the mild illness, contracted by animals exposed to swine influenza as induced by Strain 15 virus and *H. influenzae suis* Culture 451, was filtrate disease and was caused by the transfer of virus without the corresponding transfer of the organism to the exposed animals. That the organism still maintained its ability, when once established in the swine respiratory tract, of acting with the virus in inducing characteristic swine influenza was shown by the regularity with which infections could be produced by the intranasal inoculation of swine with organism and virus.

It was evident that sometime after March, 1931, when the strain of swine influenza under discussion was last known to be fully contagious, a change had taken place in one or both of the etiological agents to alter the contagious character of the complete disease in the manner described. In order to determine definitely whether the failure of *H. influenzae suis* Culture 451 to establish itself in the swine respiratory tract under conditions of infection by contact was due to some change in its own properties or to some change in the character of the virus with which it was associated, a fresh epizootic field strain of swine influenza was obtained.

Experiments with a Fresh Field Strain of Epizootic Swine Influenza

A widespread outbreak of swine influenza which occurred among the swine on exhibition at the Iowa State Fair early in September, 1932, furnished fresh infectious material. Lung, bronchial exudate, and bronchial lymph nodes from an animal killed on the 3rd day of a typical but severe illness were brought back to the laboratory on ice. Cultures, revealing large numbers of *H. influenzae suis* in the lung and bronchial exudate had been made before leaving Iowa. A normal pig inoculated intranasally with a suspension prepared from the pathological material brought back, and fortified by the addition of the cultures that had been isolated before leaving Iowa, developed characteristic swine influenza. This fresh field strain of the disease proved to be fully and typically contagious, and *H. influenzae suis* was isolated in pure primary culture from the lung and bronchial exudate of the pig infected by contact. The new virus was designated Strain 18, and the associated bacillus, *H. influenzae suis* Culture 18.

The means were now available for determining which of the two etiological components of the old stock strain of swine influenza was

TABLE I

Differences in the Contagious Character of Swine Influenza Induced by Old and Recently Procured Strains of Infectious Material

Experiment No.	Swine No.	Inoculated intranasally with		Clinical picture	Autopsy findings	H. influenzae suis in		Remarks
		Swine influenza virus, strain No.	H. influenzae suis, culture No.			Lung	Bronchial exudate	
1	1213	Infectious material from naturally occuring case of swine influenza (Strain 18—1932)		Swine influenza	Typical	Mixed culture	Mixed culture	Obtained at Iowa State Fair—Sept. 2, 1932
	1195	Contact with Swine 1213		Severe swine influenza	Moderate	Pure culture	Pure culture	
2	1212	18 (1932)	18 (1932)	Swine influenza	Extensive	Pure culture	Mixed culture	
	1251	Contact with Swine 1212		Swine influenza	Extensive	Mixed culture	Mixed culture	
3	1283	18 (1932)		Mild swine influenza	Typical	Pure culture	Pure culture	
	1286	Contact with Swine 1283		Mild swine influenza	Moderate	Absent	Mixed culture	
4	1192	18 (1932)	451 (1928)	Swine influenza	Typical	Pure culture	Pure culture	
	1250	Contact with Swine 1192		Filtrate disease	Few	Absent	Absent	
5	1282	18 (1932)	451 (1928)	Mild swine influenza	Moderate	Mixed culture	Mixed culture	
	1289	Contact with Swine 1282		Filtrate disease	Few	Absent	Absent	
6	1191	15 (1930)	451 (1928)	Mild swine influenza	Moderate	Mixed culture	Pure culture	
	1231	Contact with Swine 1191		Filtrate disease	Few	Absent	Absent	

	Pig No.	Inoculation		Condition produced	Lung lesions	Culture	Culture	Remarks
7	1135	15 (1930)	451 (1928)	Swine influenza	Typical	Pure culture	Mixed culture	
	1134	Contact with Swine 1135		Filtrate disease	Few	Absent	Absent	
8	1217	15 (1930)	18 (1932)	Swine influenza	Extensive	Pure culture	Mixed culture	
	1222	Contact with Swine 1217		Swine influenza	Extensive	Mixed culture	Pure culture	
9	1277	15 (1930)	18 (1932)	Swine influenza	Typical	Pure culture	Pure culture	
	1244	Contact with Swine 1277		Fatal swine influenza	Edematous pneumonia	Pure culture	Pure culture	Died on 2nd day of illness

responsible for the change in its contagious character.   This was done
by substituting the individual components of the freshly obtained and
fully contagious field strain of the disease for corresponding compo-
nents of the old stock strain.   Representative experiments in which this
was done are outlined in Table I.

In the experiments given in Table I, the virus used was in all instances filtered
through Berkefeld candles, adding broth cultures of *B. prodigiosus* as the test
organism.   All filtrates were cultured on media capable of supporting the growth
of both *B. prodigiosus* and *H. influenzae suis* and all virus samples recorded were
bacteriologically sterile.   The cultures of *H. influenzae suis* used in the experi-
ments were grown in sterile defibrinated horse blood at the bases of plain agar
slants, and all Strain 18 cultures were transferred frequently enough, before use,
to free them of any swine influenza virus carried over mechanically from the
infectious material used as a source.   The Strain 18 culture when administered
alone intranasally to swine was shown to be completely incapable of inducing any
clinical evidence of illness or pathological alteration detectable at autopsy.

The data presented in Table I indicate that Strain 18 virus and
Strain 18 culture, when mixed and administered intranasally to swine,
induced characteristic swine influenza, fully contagious for normal
animals.   Strain 15 virus mixed with Strain 18 culture and adminis-
tered intranasally to swine also produced a fully contagious swine
influenza.   However, when Culture 451 was substituted for Culture 18,
with either Strain 15 or 18 virus, the disease produced by intranasal
inoculation, while clinically and pathologically characteristic of swine
influenza, was not fully contagious for normal animals.   Instead of
swine influenza, these animals infected by contact developed the mild
filtrate disease.   It was thus apparent that the change in the con-
tagious character of the old stock strain of swine influenza had been
due to some biological alteration in Culture 451 and that Virus 15 was
in no way responsible.   Experiments are now being carried on to
determine some significant difference between Culture 18 and Culture
451 which will account for the latter's failure to transfer with the swine
influenza virus in contact infections.

The Production of Swine Influenza by Contact in Animals Experi-
mentally Converted into Carriers of H. influenzae suis

A small group of experiments performed earlier acquire particular
interest following establishment of the fact that the change was in the

bacterial and not the virus component of the etiological complex. These will be reported at this time because they may be of some significance in the problem of respiratory infections in general and also because they reinforce the conclusion already reached that the contact infection in animals exposed to the old stock strain of swine influenza represented an infection in which *H. influenzae suis* had not transferred with the virus.

In a preliminary experiment it had been determined that when a pig, which had been inoculated intranasally 3 days previously with *H. influenzae suis*, of itself innocuous, was given by the same route a small amount of Berkefeld filtrate of swine influenza virus, it promptly developed characteristic swine influenza. A control animal which received only the virus developed the mild filtrate disease. It was concluded from such an experiment that in carriers of *H. influenzae suis*, inoculation with the virus alone was sufficient to induce swine influenza. This fact was utilized in attempting to determine the nature of the mild illness contracted by pigs exposed to cases of the old stock strain of swine influenza. Experiments were conducted in which carriers of *H. influenzae suis* Culture 451 were placed in contact with cases produced by inoculation with the old stock strain of swine influenza. Protocols of these experiments are outlined in Table II. In two of the experiments recorded in Table II, pigs that were not carriers of the organism were included. In Experiments 2 and 3 the animals to be infected by exposure were not introduced into the pens until 24 hours after the inoculation of the animals to whose disease they were to be exposed; while in Experiment 1 the animals to be exposed were put into the pen with the infected animal soon after its inoculation.

In all three experiments carriers of *H. influenzae suis*, when exposed to the old stock strain of swine influenza, developed, after incubation periods of from 24 to 48 hours, a characteristic but unusually severe swine influenza. All three of the animals infected in this way showed at autopsy an edematous pneumonia of the type seen in severe and usually fatal cases of swine influenza. One of the animals died on the 4th day of its illness and the remaining two were extremely sick when sacrificed on the 3rd and 4th day. As in similar experiments performed earlier, the swine that were not carriers of *H. influenzae suis* developed only filtrate disease following exposure to cases of the old stock strain of swine influenza. These three experiments suggest that the disease developing in carriers of *H. influenzae suis* after contact with cases of swine influenza is more severe than that induced by

TABLE II

Contagion of Swine Influenza for Animals That Are Carriers of H. influenzae suis

Experiment No.	Swine No.	Mode of infection	Clinical picture	Autopsy findings	H. influenzae suis in		Remarks
					Lung	Bronchial exudate	
1	1125	HIS* + dried and glycerolated influenza virus i.n. † Jan. 11	Swine influenza	Typical	Mixed culture	Mixed culture	
	1123	HIS alone i.n. Jan. 9 Contact Swine 1125 Jan. 11	Negative Swine influenza	Water-logged type of pneumonia	Absent	Mixed culture	Marked pleuritis and pericarditis yielding pure cultures of HIS
	1122	Contact Swine 1125 Jan. 11	Mild filtrate disease	Few	Absent	Absent	Disease typical of that induced by virus alone
2	1063	HIS + dried and glycerolated influenza virus i.n. Jan. 18	Swine influenza	Typical	Pure culture	Mixed culture	
	1062	HIS alone i.n. Jan. 18 Contact Swine 1063 Jan. 19	Negative Swine influenza	Water-logged type of pneumonia	Absent	Pure culture	Marked pleuritis and pericarditis. Animal died on 4th day of illness

	No.	Treatment	Swine influenza	Typical	Pure culture	Mixed culture	
3	1135	IIIS + dried and glycerolated influenza virus i.n. Feb. 22	Typical		Mixed culture	Mixed culture	
	1127	IIIS alone i.n. Feb. 20 Contact Swine 1135 Feb. 23	Negative	Water-logged type of pneumonia	Mixed culture	Mixed culture	Extensive pleuritis yielding pure cultures of IIIS
	1134	Contact Swine 1135 Feb. 23	Mild filtrate disease	Few	Absent	Absent	Disease typical of that induced by virus alone

* IIIS = *H. influenzae suis* Culture 451.
† i.n. = intranasally.

intranasal inoculation with mixtures of virus and *H. influenzae suis*. With so small a series of cases no conclusion is drawn at this time.

No controls for the pathogenicity of *H. influenzae suis* alone were included in the experiments given in Table II because Culture 451, which was used, had been tested repeatedly, both in previous years and recently, and found, with the exception of a test made very shortly after its isolation in 1928, to be uniformly incapable of inducing illness that could be confused with influenza (2). It has, furthermore, been tested since the experiments recorded in Table II were conducted and found to be still completely non-pathogenic for swine. Swine that were converted into carriers of *H. influenzae suis* were under observation and exhibited no evidence of illness or elevation of temperature to a fever level not only for the 24 to 72 hours elapsing between the time of inoculation with the organism alone and their exposure to the disease, but also during the 24 to 48 hour latent periods between their exposure and the onset of their illness. They were therefore recorded as negative under the heading of "clinical picture" in Table II.

The results described above accord with the conclusion already reached that the contact infection in pigs exposed to the old stock strain of swine influenza is one in which *H. influenzae suis* is unable to establish itself with the virus in the respiratory tracts of the exposed animals. The resulting illness, identical with the filtrate disease, is due to infection with the swine influenza virus alone.

DISCUSSION

The experiments reported in this paper may prove to be of interest in the general problem of respiratory infections in that they demonstrate three types of infection possible with an etiological complex made up of a virus and a bacterium. The first of these types is that seen in the epizootic disease in which both virus and *H. influenzae suis* transfer from sick to normal animals by exposure. The second and third types, while not known to exist under field conditions, are none the less possible. They are exemplified by the experiments given in Table II. In these two types only the virus transfers by contact from sick to normal animals and the severity and type of the resultant disease are dependent upon whether or not the infected animal is a carrier of *H. influenzae suis*. If it is not, it develops only a very mild and transient illness which, however, is transmissible to other swine. If it is already a carrier of *H. influenzae suis*, infection with the virus produces true swine influenza. Thus, in a hypothetical swine popula-

tion in which some of the animals were carriers of *H. influenzae suis* of the type of Culture 451, introduction of the swine influenza virus would result in two kinds of disease clinically quite different: swine influenza in the carrier animals, and an extremely mild and poorly defined illness in the remainder. In this population it would not be impossible for a carrier to develop a fatal infection from exposure to a very slightly sick non-carrier, and, in turn, the infection transferred from the fatally ill carrier animal to one that was not a carrier might be so slight as to be scarcely recognizable. If now, in this hypothetical population, there was one animal carrying an *H. influenzae suis* of the type of Culture 18, capable of transferring with the virus by contact, the disease would undoubtedly proceed as epizootic swine influenza, infecting each new case with both virus and organism regardless of whether or not it had previously been a carrier.
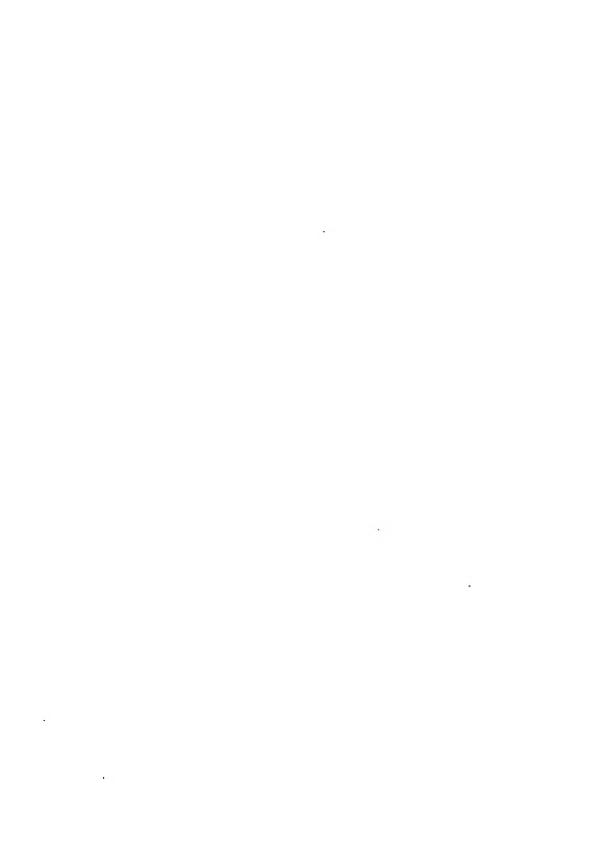
SUMMARY

A strain of swine influenza has been observed to change from a condition of full contagiousness, in which both *H. influenzae suis* and the swine influenza virus were transferred by pen contact, to one of only partial contagiousness, in which the virus alone was transferred, resulting in the mild filtrate disease instead of swine influenza in animals infected by contact. Swine that had been experimentally converted into carriers of *H. influenzae suis* developed swine influenza following contact with animals infected with the altered strain of the disease. Experiments in which the etiological components of a freshly obtained and fully contagious strain of swine influenza were substituted for the corresponding components of the altered strain of the disease revealed the fact that the change in the contagious character of the latter was due to an alteration in the bacterial component of the etiological complex and that the virus component was in no way responsible.

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ON SPECIFIC INHIBITION OF BACTERIOPHAGE ACTION BY BACTERIAL EXTRACTS*

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In a brief note dealing with the specificity of bacteriophage on organisms of the typhoid group, Hadley (3) mentioned the hypothesis that the action of phage on the sensitive bacillus is determined by the constitution of the heat-stable agglutinogens. The theme has been considered by Burnet in investigations covering the several recognized groups of the Salmonella organisms (4–6). It is generally agreed that lysis by phage requires a preliminary absorption of the active agent onto the bacterial surface, and the assumption is that the specificity of the absorption is attributable to the carbohydrate haptens which in the case of the Salmonella group of organisms, as shown by Furth and Landsteiner (7, 8), are endowed with the same specificity as the heat-stable agglutinogens. The hypothesis of Hadley and Burnet requires that the agglutinin-absorbing property of an organism and its phage-absorbing capacity run parallel.

The evidence for the hypothesis is based in the main part on direct tests of phages on representative cultures of the Salmonella group. Thus, in the experiments of Burnet made on agar plates a certain phage which lysed *B. enteritidis,* lysed also several organisms which share with the former strain the same heat-stable agglutinogens (heat-stable factors), namely *B. pullorum, B. sanguinarium, B. typhosus,* but did not act on cultures of *B. aertrycke, B. derby, B. paratyphosus* A, and *B. newport.* That the heat-labile flagellar antigens played no rôle in the specificity, was shown by the action of this *enteritidis* phage on the non-motile organisms *B. pullorum* and *B. sanguinarium.* Other phages active for *B. enteritidis* were not entirely specific but merely gave a greater incidence of reactions on the typhoid-*enteritidis* group of bacilli than on those of *paratyphosus* A or *paratyphosus* B.

Further support for the hypothesis that the specificity of phage depends upon

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* See preliminary papers (1, 2).

the same antigenic components that have affinity to antibodies, was found by Burnet in the specific behavior of rough and smooth cultures to a series of phages. In accordance with a striking change in antigenic specificity, as evidenced by both agglutinin absorption experiments and also distinct bacterial carbohydrate extracts, accompanying the change of phase from smooth to rough, there was a more or less sharp difference in the behavior of the two sorts of cultures to phages. Thus Burnet described phages which acted mainly on a smooth phase, others which lysed mainly rough derivatives of the same bacilli, and a third variety which acted on both (5).[1] There were indications also that phages sensitive for the rough organisms generally had a more extensive scope of action covering several species of the Salmonella, an observation which is in harmony with the loss of specific antigens in such strains and the presence of an antigen common to the entire group (4)[2] (6).[3]

Burnet's experiments with both the Flexner group of dysentery organisms and varieties of staphylococci are also in harmony with the view expressed above (9, 10). Of particular interest in this connection is an early observation of Marcuse (11) who described a phage active for *B. dysenteriae* Flexner Type Y, and also for certain coliform strains; only the sensitive coliform organisms, and not the resistant strains, absorbed the agglutinins for the Type Y organisms.

Burnet recognized that a number of his own observations as well as those of others were not compatible with the hypothesis under consideration.[4] In the first place, as already mentioned, some of the phages, although apparently selective for a certain group, reacted also with cultures outside of the group. Also the organisms obtained from the overgrowth after the action of phage on sensitive bacilli and in the same phase as the parent strain are resistant to lysis, although it is not possible to demonstrate a difference in the heat-stable agglutinogens in the two strains by cross-absorption of the two sorts of sera (13, 14). The lack of action of phage on such resistant cultures can be understood if these organisms fail to absorb the phage particle, presumably by virtue of a corresponding change in the specific carbohydrate complex not serologically detectable (6). While the failure of phage absorption by resistant organisms has been reported by Burnet (13), Prausnitz and Firle (15), Lepper (16), Kimura (17), and others,

[1] Burnet (5), Table 1, p. 19.
[2] Burnet (4), Table 5, p. 127.
[3] Burnet (6), Table 1, p. 654.
[4] More recently Burnet (12) has published on other aspects of the specificity of bacteriophage, using the antiphage reaction together with the various resistance groups after the method of Bail.

positive results have been obtained by some workers—Flu (18), Applemans (19), Gohs and Jacobson (20).

Should the hypothesis be correct, it ought to be possible to demonstrate specific binding of phage or inhibition of its action in suitably prepared mixtures of phage and sterile solutions of the specific soluble carbohydrate-containing extracts. Actually such experiments have been carried out by several workers with negative results (6, 15). Since a final explanation for the specificity involved in bacteriophage action is not available, a reinvestigation has seemed warranted.

Our first tests were made according to the general principle employed by Burnet; namely, by direct experimentation on a large number of smooth and a smaller number of rough Salmonella cultures by using suitable dilutions of active phages derived against representative smooth organisms of the several groups of White (22) and Kauffmann (23).[5] In contrast to the work of Burnet, our experiments were made in test-tubes. The results of these investigations with phages active against *B. paratyphosus* B (factors I and II),[5] *B. enteritidis* (factor III), *B. paratyphosus* A (factor VI) soon revealed that a clear-cut group specificity sufficient to establish the identity of cultures within any group was not readily demonstrable. The difficulties encountered were in part similar to those already mentioned; namely, a small number of cultures were totally resistant to phage action, while a larger number in our collection, especially in the typhoid-*enteritidis* group, were lysogenic and cleared spontaneously in broth. Only one of these phages (*B. paratyphosus* B) showed a tendency towards a group specificity, giving a relatively greater incidence of lytic reactions on members of the *paratyphosus* B group than on organisms of the *paratyphosus* A, hog cholera, and *newport* groups. The phage for a smooth *B. enteritidis* showed action on numerous Salmonella cultures irrespective of serological grouping, except for a few strains which resisted the action of several phages employed. Another phage active for a rough strain of *enteritidis* gave a considerable number of reactions with rough strains of many Salmonella organisms and also with numerous smooth cultures irrespective of antigenic composition.

While direct tests of phages on numerous bacilli did not yield clear-cut results in our hands, nevertheless they gave indications of some rough parallelism between phage action and antigenic composition of heat-stable agglutinogens. Tests by means of another method not yet extensively employed for the study of phage specificity, namely absorption, a procedure found to be so useful for investigations on the

[5] In this paper White's numberings of the somatic factors (heat-stable agglutinogens) are given.

specificity of antigen-antibody reactions, have thus far yielded conflicting results. Consequently for the further investigation of the question, the important experiments of Prausnitz (15) and Burnet (6), designed to demonstrate a reaction between phage and specific soluble substance, were repeated. We employed lysis in test-tubes instead of placque counts since it appears from the literature (21) that the former may be a more delicate criterion of phage activity, allowing for continuous observations on the progress of lysis. Under such conditions, as the following data show, clear-cut specific inhibition of phage action by bacterial extracts was obtained.

Description of Material

Phages.—All the phages employed in these experiments were obtained from chicken stool filtrates. The titer of the phage was increased by several successive passages in beef extract broth (pH 7.4–7.6) against the homologous organism.

Cultures.—The cultures were the same employed by Furth and Landsteiner (8) in their studies on precipitable substances of the Salmonella group and were originally obtained by them from the National Collection of Type Cultures, Lister Institute, London.

Bacterial Extracts.—The carbohydrate-containing extracts were prepared, on the whole, according to the method of Furth and Landsteiner (8). Organisms were grown in Blake bottles on beef extract agar for 48 hours at 37°C. The bacilli were taken up in 0.85 per cent sodium chloride solution, centrifuged, and the sedimented bacteria were suspended and washed in 95 per cent alcohol. The bacterial mass was extracted in boiling 95 per cent alcohol (10 cc. per bottle) and then in absolute alcohol (5 cc. per bottle). After centrifugation the alcohol-extracted bacteria were heated twice for 1 to 2 hours in a steam bath in 0.85 per cent sodium chloride solution (5 cc. per bottle). The combined saline extracts were precipitated with 3 to 4 volumes of 95 per cent alcohol. (The precipitation of proteins by acid from the saline extracts and the addition of alkali prior to flocculation by alcohol were omitted from our procedure.) The precipitates were then washed in absolute alcohol, ether and dried.

EXPERIMENTAL

The experiments were made in the following manner: the crude bacillary saline extracts of *B. dysenteriae* Shiga and *B. paratyphosus* B were dissolved in saline in sufficient amounts to make a 1:200 solution and filtered through a Seitz filter. Equal volumes (0.25 cc.) of the extracts and dilutions of phage were mixed and incubated at 37° overnight. The test to detect inhibition was made the following day by adding 4.5 cc. beef extract broth to each of the tubes together with 3 to 4 drops of a young suspension of the homologous test organisms (1 loopful of a

TABLE I, *a*

Test with B. dysenteriae Shiga

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. dysenteriae* Shiga phage							
	10^{-2}	10^{-4}	10^{-3}	10^{-6}	10^{-3}	10^{-8}	10^{-7}	10^{-10}
B. dysenteriae Shiga	+	+±	++	++	++	++	++	++
	cl	±	++	++	++++	++++	++++	++++
	cl	cl	cl	cl	++++	++++	++++	++++
B. paratyphosus B	cl	±	+	+±	+±	++	++	++
	cl	cl	cl	±	+±	+±	++++	++++
	cl	cl	cl	cl	cl	cl	++	++++
Saline solution	cl	cl	±	+±	++	++	++	++
	cl	cl	cl	cl	+±	+±	++++	++++
	cl	cl	cl	cl	cl	cl	++	++++

TABLE I, *b*

Test with B. paratyphosus B

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. paratyphosus* B phage							
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
B. dysenteriae Shiga	cl	+	+±	++	+++±	+++	+++	+++
	cl	cl	cl	cl	++	+++±	++++	++++
	cl	cl	cl	cl	cl	+	++++	++++
B. paratyphosus B	+±	+±	++±	+++±	+++	+++	+++	+++
	cl	+±	++++	++++	++++	++++	++++	++++
	cl	+	+	+±	++	++++	++++	++++
Saline solution	cl	+	+±	++	+++±	+++±	+++	+++
	cl	cl	cl	cl	++	+++±	++++	++++
	cl	cl	cl	cl	cl	+	++++	++++

Readings were made after incubation at 37° for 3, 5, and 7 hours following the addition of the test organism.

In these and in the following protocols, cl indicates clearing, tr, trace, and the signs ±, +, +±, ++, ++±, etc. indicate increasing degrees of turbidity.

young agar slant to 50 cc. broth). The mixtures were incubated and turbidity readings were made at frequent intervals in order to follow the course of the lysis. Tests were made at the same time with anti-*B. paratyphosus* B phage and with

anti-*B. dysenteriae* Shiga phage in order to make observations on the specificity of the reaction.

The results in Table I showed a distinct specific inhibition of phage action by the homologous carbohydrate-containing extract. Thus, the course of lysis was specifically delayed only for a short interval in those tubes containing the larger quantity of phage and the effect was seen only in the early readings; whereas in the tubes containing small quantities of phage, the more striking effects were observed in the later readings.

In the experiments tabulated there was little or no inhibition resulting from contact with the heterologous extract, but in several cases a slight non-specific inhibition was at times evident in the tubes containing the smaller quantities of phage. In a few instances in which this effect was observed it did not increase much on prolonged incubation and did not obscure the specificity.

The specific effect in the case of the Shiga phage was still present on the following day but in the phage for *B. paratyphosus* B overgrowth occurred rapidly in those tubes which had cleared, so that the inhibition was more or less obscured. However, the effect could still be verified even in the set of tubes which were equally turbid (for instance those containing phage 10^{-5} and 10^{-6}), by the observation that where heterologous substance was present the turbidity was due to organisms resistant to phage; in the tubes containing the homologous extract, however, the union of phage and extract completely inhibited lysis so that the bacteria still remained sensitive to fresh addition of the lytic agent.

For routine experiments, observations were made at frequent intervals until lysis in the control progressed practically to its end-point.

At this point it may be mentioned that in several experiments the specific inhibition was evident when placque counts were used as a criterion of phage action. In these tests the incubated mixtures were plated together with a few drops of a young suspension of homologous organisms.

The Effects of Time and Temperature

For further progress it seemed desirable to gather information relative to the conditions most suitable for the demonstration of the specific inhibition.

For this purpose, mixtures containing 4 volumes (1 cc.) of varying dilutions of anti-*B. dysenteriae* Shiga phage and 4 volumes (1 cc.) of crude saline extracts, were prepared in a manner similar to those of the previous experiment. These mixtures were divided into four equal parts, each tube of the series containing 0.25 cc. of phage and 0.25 cc. of the crude extract. One part was allowed to incubate overnight at 37°C., another kept overnight at room temperature, a third set for the same period in the ice chest, and the fourth was tested without previous incubation. The tests were made by adding 4.5 cc. of beef extract broth to each of the tubes followed by 3 drops of a 1 hour old broth suspension of *B. dysenteriae* Shiga. The

TABLE II

Test with B. dysenteriae Shiga*

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. dysenteriae* Shiga phage						Incubation at
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	°C.
B. dysenteriae Shiga........	±	+±	+++	++++	++++	++++	37
B. paratyphosus B.........	cl	cl	cl	+	++	++++	
B. dysenteriae Shiga........	+	+±	+++±	+++	++++	++++	22
B. paratyphosus B.........	cl	+	+±	+++±	++++	++++	
B. dysenteriae Shiga........	cl	cl	cl	+±	++++	++++	5
B. paratyphosus B.........	cl	cl	cl	+	+++	++++	
B. dysenteriae Shiga........	cl	cl	+	++	++	++++	Unincubated mixtures
B. paratyphosus B.........	cl	cl	cl	±	++	++++	

The reading recorded was made after incubation at 37° for 7 hours following the addition of the test organism.

* Since the degree of non-specific inhibition was negligible, the saline controls were omitted from the table.

tests were incubated at 37° and turbidity readings made at frequent intervals to observe the course of lysis.

Due to lack of space, one representative turbidity reading is given, save in Table I where several are presented to illustrate the general course of events. While the results are definite as shown in Table II, and in all others containing a single reading, the effects, in all cases, were clearer when the progress of lysis was observed at frequent intervals.

These tests showed that the most pronounced inhibition occurred in those mixtures incubated overnight at 37°. Practically no effect could be seen in the mixtures kept in the refrigerator. In the other two sets intermediate results were obtained, the inhibition being more distinct in the mixtures held overnight at room temperature. It is noteworthy that the inhibition was better in the unincubated mixtures (tubes with phage 10^{-4}) than in those incubated overnight in the ice box.

In similar experiments using anti-*B. paratyphosus* B phage, the results, on the whole, corresponded to those obtained with the anti-*B. dysenteriae* Shiga phage, in that the best effect was observed in the set incubated overnight at 37° and the least in the set kept at the lowest temperature. The results differed in that the tests with anti-*B. paratyphosus* B phage kept in the ice box and the series without incubation showed inhibition of lysis which was more distinct than the corresponding tests with anti-*B. dysenteriae* Shiga phage. From these observations it follows that the specific union of the anti-*B. paratyphosus* B phage and bacillary extract may be greater than that of the anti-*B. dysenteriae* Shiga phage and Shiga bacillary extract. An alternative view, however, is that the affinity between the anti-*B. paratyphosus* B phage and the homologous bacillus is weaker than the union of the anti-*B. dysenteriae* Shiga phage and the Shiga bacillus.

Another series of experiments was made with the two phages in which temperatures of 37°, 45°, and 55°C. were employed and intervals of incubation varying from 3 to 14 hours. These indicated that incubation of the mixtures at the higher temperatures did not yield a specific inhibition which was markedly better than that obtained after incubation for the same length of time at 37°. Consequently for all further experiments the routine procedure of incubation at 37° overnight was adopted. In addition these tests showed an increase in the specific inhibition with the time increment at any one temperature employed. Again the two phages behaved differently in that the degree of inhibition, especially after the shorter period of incubation, was distinctly better in the case of the anti-*B. paratyphosus* B phage.

It is still to be determined whether these conditions of time and temperature obtain, also, for other phages and extracts derived from other organisms. That the state of affairs may be somewhat different for other cases seems not unlikely in view of the differences in the

properties of the anti-*B. dysenteriae* Shiga and anti-*B. paratyphosus* B phages just discussed.

Specificity of Phage Inhibition within the Salmonella Group

It has been demonstrated in the first part of this paper that the action of the anti-*B. paratyphosus* B phage and the anti-*B. dysenteriae* Shiga phage is specifically inhibited by extracts of the homologous bacilli. These organisms, however, belong to entirely different and unrelated species. A more severe test of the specificity of the reaction could be applied to extracts of organisms and phages within the Salmonella group which, although possessing a number of substances in common (those connected with flagellar antigens, the substance G in the terminology of White (22), and some proteins studied by Furth and Landsteiner (7) and also White (24)) nevertheless fall into distinct groupings which are defined by the distribution of the various soluble specific substances of the heat-stable or somatic factors.

One series of experiments was made with extracts and phages for *B. paratyphosus* B (somatic factors I and II), and for *B. suipestifer* (somatic factor V). Both phages were specific in their reactions on the two cultures when the tests were put up in tubes. The tests were made in the usual manner, save that a trace of alkali was employed to aid solution of the extracts.

The results, Tables III, *a*, and III, *b*, show a striking specific inhibition of the two phages since the course of the lysis of the anti-*B. suipestifer* phage was distinctly retarded by the homologous extract but only slightly by an extract of *B. paratyphosus* B. On the other hand the extract of *B. suipestifer* but weakly influenced the action of anti-*B. paratyphosus* B phage which was inhibited to a considerable degree by its own extract. It is not without significance that the degree of non-specific inhibition by the extracts of the Salmonella organisms was not much greater than that by the more distantly related *B. dysenteriae* Shiga (compare with Tables I and II).

In the same set of experiments the two phages were tested also with extracts of *B. dysenteriae* Shiga, *B. paratyphosus* A, (somatic factor VI), *B. stanley* and *B. acrtrycke*, the latter two organisms sharing with *B. paratyphosus* B the same somatic factors I and II, but differing from each other by virtue of specific flagellar antigens. None of these extracts influenced the action of the anti-*B. suipestifer* phage, an

observation which is apparently in agreement with the failure of this phage to lyse any of the organisms mentioned. However, extracts of *B. stanley* and *B. aertrycke* distinctly inhibited the action of anti-*B. paratyphosus* B phage. On testing this phage directly on these two organisms it was observed that while it lysed *B. aertrycke* to a titer of 10^{-5}, it had very weak action on *B. stanley*, 10^{-1}. Consequently other experiments were made to investigate the relationship of phage

TABLE III, *a*

Test with B. paratyphosus B

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. paratyphosus* B phage						
	1×10^{-4}	1×10^{-5}	1×10^{-6}	3×10^{-6}	1×10^{-7}	3×10^{-7}	1×10^{-8}
B. suipestifer............	cl	cl	±	++	++	++++	++++
B. paratyphosus B.......	+±	+++	++++	++++	++++	++++	++++
B. dysenteriae Shiga......	cl	cl	cl	+	+++	++++	++++
Saline solution.........	cl	cl	cl	cl	++±	++±	++++

TABLE III, *b*

Test with B. suipestifer

Phage dilution incubated with 1:200 extract of	Dilutions of anti-*B. suipestifer* phage						
	3×10^{-2}	1×10^{-3}	3×10^{-3}	1×10^{-4}	3×10^{-4}	1×10^{-5}	3×10^{-5}
B. suipestifer...............	±	++	+++	+++	++++	++++	++++
B. paratyphosus B..........	cl	cl	±	+	++	++	++++
B. dysenteriae Shiga.........	cl	cl	cl	+	++	+++	++++
Saline solution.............	cl	cl	cl	+	+±	+++	++++

The reading recorded was made after incubation at 37° for 5 hours following the addition of the test organism.

titer on the living bacillus to inhibition of phage by extracts of these organisms. For this purpose two other organisms were included; namely, *B. tidy* of the same antigenic composition and a smooth resistant strain of *B. paratyphosus* B, isolated from the overgrowth following phage action on the sensitive smooth parent culture employed throughout these experiments. This organism, still resistant to phage following numerous transplants on agar over a period of 10 months after its isolation, could not be differentiated morphologically

or biochemically from the parent culture. Also cross-absorption of the two sorts of immune sera with the two organisms failed in our hands to reveal a serological difference (13, 14).

The titer of the anti-B. *paralyphosus* B phage on the homologous organism was about 10^{-8}, on B. *tidy*, 10^{-7}, B. *aertrycke* and B. *stanley*, as already mentioned, 10^{-5} and 10^{-1} respectively, and of course no lytic action at all on the resistant strain.

Crude saline carbohydrate-containing extracts of all these organisms were prepared and tested for their capacity to react with the anti-B. *paralyphosus* B phage, using the homologous culture as a test

TABLE IV

Test with B. paralyphosus B

Phage dilution incubated with 1:200 extract of	Dilutions of anti-B. *paralyphosus* B phage						
	10^{-2}	10^{-2}	10^{-4}	10^{-5}	10^{-5}	10^{-7}	10^{-8}
B. *paralyphosus* B (sensitive)	cl	+±	+++±	++++	++++	++++	++++
B. *paralyphosus* B (resistant)	cl	+	++	++++	++++	++++	++++
B. *aertrycke*	cl	+	+±	++++	++++	++++	++++
B. *stanley*	+	++±	++++	++±	++±	++++	++++
B. *tidy*	cl	+	+±	++++	++++	++++	++++
B. *dysenteriae* Shiga	cl	cl	cl	cl	+	++±	++++
Saline solution	cl	cl	cl	cl	+	++±	++++

The reading recorded was made after incubation at 37° for 5 hours following the addition of the test organism.

organism. With regard to the collection of resistant organisms for the preparations of the extracts, preliminary tests made on each individual Blake bottle, showed that the bacilli grown under such conditions retained their characteristic property.

The results of the tests (Table IV) demonstrate a specific inhibition by extracts of all organisms containing the factors I and II.[6] The most intense effect was exhibited by an extract of B. *stanley*. The extracts of the four other organisms gave effects which did not differ

[6] In these and other experiments with the anti-B. *paralyphosus* B phage, specific inhibition was evident also when B. *tidy* and B. *aertrycke* were employed as test organisms.

much from each other, the *acrtrycke* extract giving the weakest inhibition.[7] Certainly the order of activity of the extracts does not run parallel to the titer of the phage against the living bacillus since the *stanley* strain was acted on only to a titer of 10^{-1}. Additional support for this view is furnished by the behavior of the resistant strain, the extract of which gave a pronounced inhibition. The failure to establish a relationship between phage inhibition by extracts and the phage titer against the living bacillus suggested other ideas, for instance the phage-absorbing capacity of the whole bacillus, the precipitating titer of the bacillary extracts, or both. Decisive evidence to form the basis for definite conclusions as to any correlation of other functions with inhibition is still wanting and experiments are under way to supply information on these issues. However, it may be stated that the *stanley* strain which gave the most intense inhibition also gave the highest precipitin titer; extracts of the resistant organism which gave distinct but somewhat weaker precipitin reactions than that of the sensitive strain, also gave weaker inhibition reactions.

In the course of these experiments the anti-*B. dysenteriae* Shiga phage was tested for inhibition after contact with extracts of eight different organisms belonging to the Salmonella group and also with its homologous extract and only the latter gave inhibition.

That the phage-absorbing capacity of the whole bacillus may play a rôle is indicated in a number of preliminary experiments, which show a distinct absorption of phage by resistant organisms obtained by fishing a single smooth colony derived from the overgrowth after action of anti-*B. paratyphosus* B phage on several strains having factors I and II (*paratyphosus* B, *stanley*, *acrtrycke*, and others). These tests, carried out with the same technique which proved effective for the demonstration of inhibition of phage by bacillary extracts, were made in the following manner.

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[7] No final attempts have been made as yet to determine how sensitive the reaction is, but in a preliminary way it can be stated that in one of the earliest experiments as little as 1:100,000 dilution of the crude *acrtrycke* extract—the weakest inhibitor—showed a distinct specific inhibition of the anti-*B. paratyphosus* B phage. Probably the reaction has about the same order of sensitivity as that of the precipitin antibodies.

The 24 hours' growth of bacilli from one agar slant was suspended in 5 cc. saline and killed by heating at 70° for 1 hour. The suspension was diluted ten times in saline and 0.25 cc. was allowed to incubate overnight at 37° with an equal volume of varying concentrations of phage. The mixture caused no turbidity on dilution with 4.5 cc. broth the following day when the test was made.

A typical protocol showing the absorption of phage by heat-killed sensitive and resistant *paratyphosus* B organisms is presented in Table V.

While in these instances there was undoubtedly a fixation of phage by resistant organisms, the objection may be raised that the absorption was due to the splitting off of a small number of sensitive bacteria.

TABLE V

Absorption of Anti-B. paratyphosus B Phage by Heat-Killed Bacilli
Test with B. paratyphosus B

Phage dilution incubated with a suspension of heat-killed	Dilutions of anti-*B. paratyphosus* B phage					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
B. *paratyphosus* B (sensitive)....	+	++++	++++	++++	++++	++++
B. *paratyphosus* B (resistant)....	±	+	++	++++	++++	++++
B. *dysenteriae* Shiga............	cl	cl	cl	±	±	++++
Saline solution.................	cl	cl	cl	tr	tr	++++

The reading recorded was made after incubation at 37° for 5 hours following the addition of the test organism.

However, the several resistant strains when tested with undiluted phage both on plates and in test-tubes showed no evidence of lysis.

However, organisms resistant to other anti-Salmonella phages derived from the overgrowth by isolation of a single smooth colony, absorbed little or no phage. This controversial question requires investigations taking into account all known criteria for resistance.

DISCUSSION

In these experiments evidence is presented that extracts of organisms containing carbohydrate material unite specifically with bacteriophage as indicated by inhibition of the lytic effect on young susceptible growing organisms. Apparently the affinity of the phage for the extract is weak and probably weaker than that of the phage for the

test organism. This fact may perhaps serve to explain the previous negative reports in such experiments. Indeed, it is difficult to determine the relative affinity of phage for a solution as compared to the affinity for a formed element.

As for the nature of the reaction, very little can be said at present except that no turbidities or precipitates have been observed in mixtures giving specific inhibition. Also such mixtures in a few experiments failed to fix complement. More progress in this direction could be made if some suitable material were found with no affinity to either the phage or extract, to be used as an indicator for the supposed specific reaction product.

The experiments suggest that the specificity of the reaction may be connected with those specific soluble substances which define the heat-stable agglutinogens. By means of this reaction, extracts derived from organisms related to B. paratyphosus B have been differentiated from the extracts of B. suipestifer. Reasoning by analogy from the work of Furth and Landsteiner, the substances involved are probably carbohydrate in nature. Indeed in a few experiments the specific phage inhibition (anti-B. paratyphosus B phages and anti-B. dysenteriae Shiga phages) was obtained also with the same substances boiled in alkali and subsequently neutralized. Certainly in these instances neither the substances connected with flagellar antigens nor the proteins played a rôle in the reaction. These experiments should be extended to other phages and substances to determine whether or not the reaction will define the remaining somatic factors of the Salmonella group and other species of bacteria in general. Perhaps investigations along this line may throw considerable light on the question of polyvalent phages (25).

In any event the demonstration of a reaction between phage and solutions of bacillary products makes it possible to study the chemistry of these products as disclosed by the effects of purification, fractionation, and hydrolysis, in terms of bacteriophage reaction and to investigate whether the findings will parallel those recently obtained on the chemistry of antigens in relation to the precipitating antibodies.

In view of our experience with phages and extracts of B. paratyphosus A and B. typhosus, it seems possible that the reaction may not be readily demonstrable in all instances. In the case of the

phage for *B. typhosus* specific inhibition has so far not been obtained and only a weak specific reaction could be demonstrated with the anti-*paratyphosus* A phage. Perhaps the most suitable conditions of time and temperature have not been as yet determined for these cases. That various phages may not be influenced in entirely the same way by time and temperature has already been indicated (see page 220). Another possibility to be considered is that the specific soluble substances mentioned above are not the only ones having an affinity for the phage. In these instances in which marked specific inhibition has not yet been demonstrated, and perhaps also in cases of those phages that have a wide scope of action covering organisms with various somatic factors, possibly the proteins described by Furth and Landsteiner and White (24) or other carbohydrates are involved (see Bronfenbrenner (25)).

The inhibition of phage by soluble bacillary products recalls some serological reactions dealing with inhibition of antibody action. Several such instances recently studied are: first, that of the haptens, thoroughly investigated by Landsteiner and van der Scheer (the inhibition of the precipitin for the coupled protein antigen by the hapten (26)); second, the inhibition of the precipitin reaction by fractions of low molecular weight derived from the specific soluble substance of Pneumococcus Type III (Heidelberger and Kendall (27)); and finally a group of cases in which the action of isoagglutinins is specifically inhibited by soluble body fluids, such as urine or saliva.

SUMMARY

1. Experiments are presented demonstrating specific inhibition of phage by soluble products of bacteria.

2. The inhibition proceeds more rapidly when the phage and bacterial extracts are incubated at 37° than at ice box temperature.

3. The specificity of the reaction in the instances studied is probably connected with the presence of specific soluble carbohydrates.

4. A reaction is available for the study of the chemistry of bacillary antigens in terms of bacteriophage.

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THE RELATION OF COAT COLOR TO THE SPONTANEOUS INCIDENCE OF MAMMARY TUMORS IN MICE*

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This communication records and discusses the spontaneous incidence of mammary tumors in yellow and non-yellow mice. The animals comprise the first hybrid (F_1) and second hybrid (F_2) generations derived from a cross between two inbred strains. The emphasis is primarily physiological. The genetic interpretation of the results is reserved for later publication.

Material

The two parental strains are as follows:

1. Dilute Brown.—This strain has been closely inbred since 1909. It has been used for many genetic experiments. Papers dealing with its tumor-producing characteristics and general physiology of reproduction have been published by Murray (1927, 1928, 1934). The strain is characterized by a high incidence of mammary tumors which are either adenomas or adenocarcinomas. Sarcomas or tumors of other organs than the mammary gland are relatively very infrequent. The strain is homozygous for color and differs from the wild house mouse by three recessive Mendelian factors.

$$a^s = \text{non-agouti or self color.}$$
$$b^r = \text{brown rather than black pigmentation.}$$
$$d^b = \text{blue dilution.}$$

2. Yellows (Dunn's Derivative of Brooke's English Stock).—These mice are less inbred than the foregoing stock. They have, however, been carried on by brother-to-sister mating for approximately 7 years. Like all yellows they are heterozygous for the factor A^y which produces yellow coat color and which is an allelomorph in the agouti series. They are also heterozygous for the factor for black pigmentation B. They are homozygous for the factor for density of pigmentation D. They are thus $A^y a^s B b^r D D$ in constitution.[1]

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* A brief preliminary note has been published by the writer and McPheters (1932).

[1] The behavior of the gene producing yellow coat color in mice is peculiar. It has been described by Castle and Little (1910), Kirkham (1917), Little (1919) and Danforth (1927).

An F_1 generation produced by a cross between these two races will consist of four types:

$$A^y a^s Bb^r Dd^b = \text{black-eyed yellows.}$$
$$A^y a^s b^r b^r Dd^b = \text{brown-eyed yellows.}$$
$$a^s a^s Bb^r Dd^b = \text{blacks.}$$
$$a^s a^s b^r b^r Dd^b = \text{browns.}$$

The yellows will appear in approximately equal numbers with the non-yellows. The blacks and browns and the black-eyed and brown-eyed yellows will in each case bear to one another a numerical relationship which will be determined by the animals picked by chance as representatives of the yellow strain to produce the F_1 generation.

The F_2 generation was produced by four types of matings of F_1 animals.

1. Yellow × yellow..................... $A^y a^s b^r b^r Dd$ × $A^y a^s b^r b^r Dd$
2. Yellow ♀ × brown ♂............... $A^y a^s b^r b^r Dd$ × $a^s a^s b^r b^r Dd$
3. Brown ♀ × yellow ♂............... $a^s a^s b^r b^r Dd$ × $A^y a^s b^r b^r Dd$
4. Brown × brown...................:.... $a^s a^s b^r b^r Dd$ × $a^s a^s b^r b^r Dd$

All animals carrying the gene B for black pigmentation were discarded before F_1 parents to produce the F_2 generation were selected. This was done to simplify the genetic situation and to reduce the number of genetic combinations possible in F_2.

To summarize then, the material consists of virgin females produced as an

(a) F_1 generation in a cross of dilute brown females (from an inbred high cancer strain) and yellow males (from a distinct inbred strain, relatively low in mammary tumors and high in sarcomas);

(b) F_2 generation from various types of matings of F_1 animals from the above cross.

All animals included in this study were produced from carefully controlled individual matings. All those included also lived longer than the earliest age at which a tumor appeared in their respective generations.[2]

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[2] The method by which individuals are listed as being of cancer age is as follows: A distribution of the animals with mammary tumors according to their age at death is made. This distribution shows a minimum age at which death of a mouse with mammary tumor is recorded. Any mouse dying before this age is excluded from the group on which a study of the incidence of cancer is based. This method is entirely consistent with that used for calculating the relative numbers of animals which are to be properly included in any recordable group. Thus in calculating the sex ratio of mice at birth the embryos that have died *in utero* cannot be included. They have not reached the critical age at which the character under observation can be recorded. Or again if we are recording the ratio of normal to hairless mice in a given generation none can be safely classified until they are over 3 weeks old since it is at that age approximately that the points of distinction between hairless and normal animals first become noticeable. The classification of tumor and non-tumor cannot therefore be attempted before the mice reach an age at which it is possible physiologically for the individual to be of either type

TABLE I

F_1 Mammary Tumor

Yellow			Non-yellow		
Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis
	days			days	
1966	732	Adenocarcinoma	1854	482	Adenoma
2162	308	"	1858	517	Medullary carcinoma-adenocarcinoma
2248	439	Adenoma			
3412	268	"	1859	518	Cyst adenocarcinoma
3455	647	Adenocarcinoma	1865	517	Adenocarcinoma
3753	340	"	1866	530	"
3762	404	"	2191	472	Papillary adenocarcinoma
3764	404	Cyst adenocarcinoma	2535	392	Adenocarcinoma
3982	404	Adenoma	2571	497	Adenoma-adenocarcinoma
3983	466	" in transition	2572	530	Adenoma
3985	403	Adenocarcinoma	2591	418	Adenocarcinoma
3986	355	"	2611	447	Cyst adenoma
3988	433	Medullary carcinoma	3203	520	Medullary carcinoma
4944	387	Adenocarcinoma	3204	551	Adenocarcinoma
4945	381	Adenoma in transition	3206	670	Adenoma, precancerous
4973	439	Adenoma-adenocarcinoma	3299	727	Cyst adenoma in transition
5004	383	Adenocarcinoma	3380	457	Adenoma
5082	267	"	3453	486	Adenocarcinoma
5083	267	"	3457	453	"
5288	538	Medullary carcinoma	3458	440	"
5531	242	Adenocarcinoma	3473	713	Adenoma-adenocarcinoma
5664	326	"	3756	781	Adenocarcinoma
			3765	467	Adenoma-adenocarcinoma
			3957	962	Cyst adenoma in transition
			3984	522	Adenoma-adenocarcinoma
			4110	421	Medullary carcinoma
			5003	383	Adenoma-adenocarcinoma
			5084	659	Adenocarcinoma
			5310	495	"
			5358	484	Adenoma-adenocarcinoma
			5467	576	Adenocarcinoma
			5468	485	"
			5532	412	"
			5533	412	Adenoma-adenocarcinoma
			5662	497	Adenoma
			6215	395	Adenoma-adenocarcinoma
Mean..	401.0		Mean..	521.0	

TABLE II

F_1 Miscellaneous Tumor

Yellow			Non-yellow		
Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis
	days			days	
1970	315	Hemangioma	1802	881	Liposarcoma infiltrating muscle
2542	991	Spindle cell sarcoma, uterus and liver	1804	1101	Papillary carcinoma, lung
			2185	884	Spindle cell carcinoma, leg and pancreas
3298	708	Neurofibrosarcoma			
4602	535	Leukemic lymphosarcoma	2189	774	Fibrosarcoma
4603	416	Lymphosarcoma	3484	820	Spindle cell sarcoma
4646	867	Leukemic lymphosarcoma			
5002	547	Lipoma			
5806	842	Hemangioma cavernosum			
Mean	653.0		Mean	892.0	

TABLE III

F₁ Non-Tumor

Yellow		Non-yellow	
Mouse No.	Age at death	Mouse No.	Age at death
	days		*days*
1813	300	1803	792
1814	469	1857	298
1856	530	2154	288
1867	465	2190	816
1873	1055	2371	768
1874	862	2375	984
1965	649	2495	1101
1967	979	2497	811
2184	869	2536	927
2251	305	3456	1223
2380	301	3649	520
2381	277	3760	997
2498	1018	5289	913
2502	272	5663	946
2537	271		
2573	392		
2613	912		
3552	1037		
3553	891		
3650	592		
3959	402		
4647	724		
4648	716		
4653	759		
4654	794		
5282	638		
6212	574		
Mean	632.0	Mean.................	813.0

TABLE IV

Color of progeny	Total No.	Mammary tumor	Miscellaneous tumor	No tumor	Per cent with mammary tumor	Per cent with miscellaneous tumor	Per cent with no tumor
Yellow............	57	22	8	27	38.6	14.0	47.4
Non-yellow........	54	35	5	14	64.8	9.3	25.9
Total............	111	57	13	41	51.4	11.7	36.9

RESULTS

F₁ Generation

There were 111 virgin female F₁ generation mice which lived to "tumor age." Of these 57 were yellow and 54 non-yellow. Of the

TABLE V

F₂ Yellow × Yellow. *Mammary Tumor*

Yellow			Dilute yellow			Brown			Dilute brown		
Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis
	days			days			days			days	
1215	256	Medullary carcinoma	1201	776	Carcinoma	935	391	Adenocarcinoma	928	335	Cyst adenocarcinoma
1276	828	Adenocarcinoma	1357	312	Adenoma	1206	653	"	2076	415	Medullary carcinoma
1320	272	"	2887	600	"	1207	729	Adenoma			
1101	457	Medullary carcinoma	2888	771	Fibroadenoma, adenocarcinoma, medullary carcinoma	1237	671	Adenocarcinoma			
1171	336	Adenoma				1312	421	Medullary carcinoma			
1617	485	"				1356	484	"			
1681	415	Adenocarcinoma				1403	445	"			
1931	327	"				1463*	581	Cyst adenoma			
1916	891	"				1466a	689	Adenocarcinoma			
2073	618	"				1513	509	Adenoma			
2071	351	"				2553	366	Adenocarcinoma			
2075	452	Medullary adenocarcinoma				2891	741	"			
2350	331	carcinoma				3477	670	"			
2890	286	Adenocarcinoma				3561	339	Medullary carcinoma			
3111	771	"				3602	478	Cyst adenoma			
3590	432	"				3621	533	Adenoma			
3620	301	Adenoma									
3814	569	Adenocarcinoma									

* See also miscellaneous tumor.

TABLE VI

F₂ Yellow × Yellow. Miscellaneous Tumor

Yellow			Dilute yellow			Brown			Dilute brown		
Mouse No.	Age at death *days*	Diagnosis	Mouse No.	Age at death *days*	Diagnosis	Mouse No.	Age at death *days*	Diagnosis	Mouse No.	Age at death *days*	Diagnosis
1405	383	Lipoma	1343	776	Liposarcoma	1463*	581	Lymphosarcoma	1411	921	Sarcoma-carcinoma of uterus
1514	603	Fibroma	1345	641	Adenoma, liver	5114	637	Fibroma, subcutaneous	1685	730	Spindle cell sarcoma vagina
5116	748	Adenoma, liver	2395	540	Lymphosarcoma				1934	820	" " sarcoma-liposarcoma

* See also mammary tumor.

yellows, 22 had mammary tumors, 8 had tumors of other types and 27 had no tumor.   Of the non-yellows 35 had mammary tumors, 5 had tumors of other types and 14 had no tumors.

Table I lists those mice having mammary tumors and gives the ledger number of the mouse; its age, in days, at death; and its diagnosis.

Table II gives the same data for non-mammary, or miscellaneous tumor animals.

TABLE VII

F₂ Yellow × Yellow.   No Tumor

Yellow		Dilute yellow		Brown		Dilute brown	
Mouse No.	Age at death	Mouse No.	Age at death	Mouse No.	Age at death	Mouse No.	Age at death
	days		days		days		days
839	423	926	774	1203	809	986	395
987	856	1406	795	1235	941	1199	644
1205	646	1520	507	1466	322	1470	724
1410	320	1681	612	1472	522	1515	629
1468	617	1683	665	1516	554	1576	973
1512	791	1935	600	1988	870	1990	765
1574	456	2057	856	1989	797		
1679	603	2802	603	2072	687		
1680	1224	3110	953	2550	545		
1885	708	3619	499	3582	572		
1987	451	4804	330	3584	686		
2059	727						
2351	426						
2397	718						
2806	648						
3587	365						
3812	417						

Table III gives the age at death and the ledger number of the non-tumor animals in the F₁ generation.

Table IV is a condensation of information concerning the F₁ generation.

F₂ Generation

The incidence of tumors in the F₂ generation may next be considered. There are, as before stated, four types of F₁ mating which were used to produce F₂ young.   These are as follows:

    (*a*) Yellow × yellow.
    (*b*) Yellow ♀ × non-yellow ♂.
    (*c*) Non-yellow ♀ × yellow ♂.
    (*d*) Non-yellow × non-yellow.

TABLE VIII

F_2 Yellow ♀ × Non-Yellow ♂. Mammary Tumor

Yellow			Dilute yellow			Brown			Dilute brown		
Mouse No.	Age at death (days)	Diagnosis	Mouse No.	Age at death (days)	Diagnosis	Mouse No.	Age at death (days)	Diagnosis	Mouse No.	Age at death (days)	Diagnosis
845	368	Adenocarcinoma	1794*	728	Adenoma	844	582	Cyst adenoma	2098	656	Adenocarcinoma
866	392	Medullary carcinoma	2479	389	Adenocarcinoma	878	567	Adenoma	2113	708	Medullary carcinoma
905	335	Adenocarcinoma				891	452	Medullary carcinoma	2115	641	Papillary cyst adenocarcinoma
906	335	"				909	664	Adenocarcinoma	2304	376	Adenocarcinoma
990	448	Medullary adenocarcinoma				1788	534	Adenoma-adenocarcinoma	2568	709	"
1122	460	Adenocarcinoma				1791	679	Adenoma	3713	563	Medullary carcinoma
1790	563	Cyst adenoma				2114	561	Adenocarcinoma	3717	560	Cyst adenoma
2116	424	Adenocarcinoma				2478	770	"	4907	466	" adenocarcinoma
2476	744	"				2570	608	Adenoma			
2480	290*	"				2903	474	Papillary adenocarcinoma			
2930	710	"				3245	545	Adenocarcinoma			
3527	475	"				3529	670	Cyst adenoma			
4906	466	"				3715	583	Adenocarcinoma			
						3917	730	Medullary carcinoma			

* See also miscellaneous tumor.

A list of animals from each of these matings is given in Tables V to XVI inclusive.

TABLE IX

F₂ Yellow ♀ × Non-Yellow ♂. Miscellaneous Tumor

Yellow			Dilute yellow			Brown		
Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis
	days			days			days	
2196	804	Liposarcoma	1794*	728	Lymphosarcoma	897	728	Lutein cell tumor, ovary
			2201	809	Liposarcoma	1789	534	Spindle cell sarcoma

* See also mammary tumor.

TABLE X

F₂ Yellow ♀ × Non-Yellow ♂. No Tumor

Yellow		Dilute yellow		Brown		Dilute brown	
Mouse No.	Age at death	Mouse No.	Age at death	Mouse No.	Age at death	Mouse No.	Age at death
	days		days		days		days
522	757	840	567	520	519	868	935
524	687	901	573	523	439	2197	694
841	487	902	710	843	1009	2203	430
846	719	992	725	860	672	2481	773
865	762	2067	781	867	718	3087	431
881	870	2095	820	882	514	3246	559
884	367	3243	388	883	539		
892	577	3712	771	886	656		
1125	699			899	752		
1126	948			2065	841		
2301	746			2070	863		
2906	733			2302	764		
3088	371			2569	805		
3093	389			3093	549		
3716	628			3095	524		
4849	426			3528	784		
4850	486			4863	766		

Table V gives the distribution of mammary tumors in the four color classes of virgin female F₂ young obtained in the (a) yellow × yellow series.

Table VI does the same for miscellaneous tumors.

Table VII gives the same data on non-tumor animals.

TABLE XI

F₂ Non-Yellow ♀ × Yellow ♂. Mammary Tumor

Yellow

Mouse No.	Age at death (days)	Diagnosis
876	567	Adenoma
962	365	Adenocarcinoma
1001	436	Adenoma
1117	382	Medullary carcinoma
1281	669	Adenocarcinoma
1477	241	Medullary carcinoma
1996	427	Adenocarcinoma
2088	467	Medullary carcinoma-adenocarcinoma
2851	688	Adenocarcinoma
3341	317	Medullary carcinoma
3499	718	Fibroadenoma
4178	332	Adenocarcinoma
4182	282	Medullary adenocarcinoma
4204	705	Fibrous carcinoma
4556	398	Adenoma-adenocarcinoma

Dilute yellow

Mouse No.	Age at death (days)	Diagnosis
1928	470	Adenocarcinoma
1929	380	Medullary carcinoma
2086	509	Adenocarcinoma
2093	608	"
3435	563	Adenoma
3495	620	Carcinoma

Brown

Mouse No.	Age at death (days)	Diagnosis
488	548	Adenocarcinoma
806	547	Adenoma
808	618	Adenocarcinoma
877	414	"
999	563	Adenoma
1114	326	Medullary carcinoma
1118*	442	" adenocarcinoma
1479	658	Adenocarcinoma
1485	581	"
1496	440	Medullary carcinoma
1506	578	Adenocarcinoma
1610	651	Adenoma
1611	651	"
1613	255	Adenocarcinoma
1973	633	Adenoma
1976	510	Papillary cyst adenoma
1978	969	Adenocarcinoma
2090	696	"
2846	349	Medullary adenocarcinoma
3145	391	carcinoma
3194	443	Adenocarcinoma
3196	514	"
3198	546	Adenoma
3492	671	Adenocarcinoma
3494	589	Adenoma
3498	706	Adenocarcinoma
3592	665	Medullary carcinoma
3923	694	Adenocarcinoma
4207	581	Adenoma
4211	517	"
4549	357	Papillary adenoma
1552	617	Medullary carcinoma
1553	639	Adenoma

Dilute brown

Mouse No.	Age at death (days)	Diagnosis
1265	359	Medullary adenocarcinoma
1333	877	Adenocarcinoma
1505	717	Adenoma
3197	573	Medullary carcinoma
3501	771	Adenocarcinoma
3593	533	"
3921	719	Adenoma
3924	435	Adenocarcinoma
4183	579	"

* See also miscellaneous tumor.

Mouse 1463 brown had both a mammary tumor (cyst adenoma) and a lymphosarcoma. It is therefore included in both Tables V and VI.

TABLE XII

F₂ Non-Yellow ♀ × Yellow ♂. Miscellaneous Tumor

Yellow			Dilute yellow			Brown		
Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis
	days			days			days	
1279	666	Lymphatic leukemia	4181	819	Adenoma, liver	683	738	Round cell sarcoma
1332	802	Round cell sarcoma, ovaries and oviducts				1113	705	Choriocarcinoma, ovary
						1118*	442	Papilloma, vagina
1501	724	Leukemic lymphosarcoma				1980	578	Leukemic lymphosarcoma
						2801	618	Polyploid epithelioma, eyelid
1938	701	Adenoma, liver						

* See also mammary tumor.

TABLE XIII

F₂ Non-Yellow ♀ × Yellow ♂. No Tumor

Yellow		Dilute yellow		Brown		Dilute brown	
Mouse No.	Age at death	Mouse No.	Age at death	Mouse No.	Age at death	Mouse No.	Age at death
	days		days		days		days
684	928	456	520	678	734	1937	846
952	456	680	543	872	695	1999	391
998	581	996	493	950	452	2386	827
1065	705	1607	596	951	527	3496	628
1066	590	1617	651	1282	791		
1478	761	1649	816	1483	560		
1616	854	1771	414	1484	657		
1653	677	1925	505	2383	872		
1979	531	1997	858	3101	954		
2055	441	2092	695	3105	423		
3201	526	2437	341	3106	666		
3343	437	4554	639	3143	672		
3436	635			3345	328		
3918	587			4184	559		
4179	264			4557	587		
4180	841			4786	845		
4205	531			4787	840		
4208	799						
4213	538						
4785	451						

Tables VIII to X give the same three groups of animals obtained from matings of the (*b*) yellow ♀ × non-yellow ♂ type. Mouse 1794

TABLE XIV

F_2 Non-Yellow $\times$ Non-Yellow. Mammary Tumor

Brown			Dilute brown		
Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis
	days			days	
482	499	Medullary carcinoma	508	586	Adenoma
497	633	Adenocarcinoma	1049	881	Adenocarcinoma
504	686	"	1052	359	"
812	735	Medullary carcinoma	1053	604	Adenoma
813	370	Adenocarcinoma	1057	383	Medullary adenocarcinoma
820	691	Adenoma	1706	537	Adenoma
821	528	Adenocarcinoma	1747	644	"
918	609	"	1751	585	Adenocarcinoma
1048	831	Medullary carcinoma	2164	710	Adenoma in transition
1063	498	Adenocarcinoma	2526	610	Adenoma
1347	501	"	3213	274	Cyst adenocarcinoma
1348	783	Adenoma	4825	478	Adenoma
1709	466	"			
1712	323	Adenoma-adenocarcinoma			
1753	430	Adenocarcinoma			
1754	521	Adenoma			
2080	509	Adenocarcinoma			
2165	673	"			
2166	443	"			
2174	380	"			
2545	625	Cyst adenoma			

TABLE XV

F_2 Non-Yellow $\times$ Non-Yellow. Miscellaneous Tumor

Brown			Dilute brown		
Mouse No.	Age at death	Diagnosis	Mouse No.	Age at death	Diagnosis
	days			days	
1059	630	Lymphosarcoma	509	522	Spindle cell sarcoma
2167	636	Lipoma	1020	341	" " "
			2451	772	Liposarcoma-adenoma, ovary

TABLE XVI

F_2 Non-Yellow $\times$ Non-Yellow-Non-Tumor

Brown		Dilute brown	
Mouse No.	Age at death	Mouse No.	Age at death
	days		days
483	490	511	661
822	713	851	642
923	596	917	865
1058	992	924	670
1350	796	1061	815
1749	390	1760	430
2084	629	2078	644
2104	576	2163	681
2107	670	2528	585
2171	776	2530	397
2529	404	3208	529
2532	905	3571	557
2544	854	3572	679
2773	688	4824	715
2778	675		
3211	484		
3212	529		
3568	535		

TABLE XVII

F₂ Generation

Type of F₁ mating	Color of progeny	Total No.	No. with mammary tumor	No. with miscellaneous tumor	No. without tumor	Per cent of total having mammary tumor	Per cent of total having miscellaneous tumor	Per cent of total having no tumor
Yellow × yellow	Yellow	38	18	3	17	47.4	7.9	44.7
	Dilute yellow	18	4	3	11	22.2	16.7	61.1
	Brown	28	16*	2*	11	57.1	7.1	39.3
	Dilute brown	11	2	3	6	18.2	27.3	54.5
	Total.........	95	40	11	45	42.1	11.6	47.4
Yellow ♀ × non-yellow ♂	Yellow	31	13	1	17	41.9	3.2	54.8
	Dilute yellow	11	2*	2*	8	18.2	18.2	72.7
	Brown	33	14	2	17	42.4	6.1	51.5
	Dilute brown	14	8	0	6	57.1	0.0	42.9
	Total.........	89	37	5	48	41.6	5.6	53.9
Non-yellow ♀ × yellow ♂	Yellow	39	15	4	20	38.5	10.3	51.3
	Dilute yellow	19	6	1	12	31.5	5.3	63.2
	Brown	54	33*	5*	17	61.1	9.3	31.5
	Dilute brown	13	9	0	4	69.2	0.0	30.8
	Total.........	125	63	10	53	50.4	8.0	42.4
Non-yellow × non-yellow	Brown	41	21	2	18	51.2	4.9	43.9
	Dilute brown	29	12	3	14	41.4	10.3	48.3
	Total.........	70	33	5	32	47.1	7.1	45.7
All F₁ matings	Yellow	108	46	8	54	42.6	7.4	50.0
	Dilute yellow	48	12*	6*	31	25.0	12.5	64.6
	Brown	156	84†	11†	63	53.8	7.1	40.4
	Dilute brown	67	31	6	30	46.3	8.9	44.8
	Total.........	379	173‡	31‡	178	45.6	8.2	47.0
All F₁ matings	All yellows	156	58*	14*	85	37.2	9.0	54.5
	All non-yellows	223	115†	17†	93	51.6	7.6	41.7
	Total.........	379	173	31	178			
All F₁ matings	All intense	264	130†	19†	117	49.2	7.2	44.3
	All dilute	115	43*	12*	61	37.4	10.4	53.0
	Total.........	379	173	31	178			

* One animal had both types of tumor.
† Two animals had both types of tumor.
‡ Three animals had both types of tumor.

dilute yellow had both an adenoma of the mammary gland and a lymphosarcoma. It is therefore included in both Tables VIII and IX.

Tables XI to XIII list the animals obtained from matings of the (c) non-yellow ♀ × yellow ♂ type. Mouse 1118 brown had a medullary adenocarcinoma of the mammary gland and a papilloma of the vagina. It is included in both Tables XI and XII.

Similar data for matings of type (d) non-yellow × non-yellow are given in Tables XIV to XVI.

The total of 379 virgin females of the F_2 generation is presented in Table XVII.

DISCUSSION

The discussion of the results obtained naturally focusses on a comparison of certain groups of animals.

One of these is the contrast between yellows and non-yellows. In the F_1 generation it was shown (Table IV) that 38.6 per cent of the yellow animals developed mammary tumors while 64.8 per cent of the non-yellows did so. This difference is 4.1 times its probable error and is therefore almost certainly significant.

Confirmatory evidence is given by the F_2 generation. Table XVII shows that 37.2 per cent of the 156 yellows grew mammary tumors while 51.6 per cent of the 223 non-yellows did so. The difference in this case also happens to be 4.1 times its probable error.

If the figures for the two generations are combined the percentages are as follows. Yellows 37.6 per cent mammary tumor, non-yellows 54.2 per cent mammary tumor. The difference is 5.7 times its probable error.

It may therefore be concluded that yellow mice are less likely than are non-yellows to grow mammary tumors.

It is interesting to note that no such difference, between the two color types, is found when the incidence of tumors of types other than mammary is combined and is considered. Here the two percentages are yellows 10.3, non-yellows 7.9, a difference of only 2.4 per cent which is certainly not significant in the number of mice available. It happens that the forty-four non-mammary tumors are equally divided between yellows and non-yellows.

The reason for the difference in mammary tumor incidence between yellows and non-yellows may next be considered. Some factors may

be definitely eliminated. Among these is the possibility of genetic linkage. Had the F_2 generation alone showed a higher incidence of mammary tumors in non-yellows, linkage might have been considered a possible explanation. Since, however, the F_1 generation shows a difference of the same sort, linkage between non-yellow and high incidence of mammary tumors is out of the question. Similarly there can be no question of sex linkage because the F_1 generation was produced by crossing females of the high mammary tumor (dilute brown) strain with males from the yellow strain. Thus the F_1 females all carried the same x-chromosome combination and offer no grounds for expecting a difference in tumor incidence such as was actually observed.

There are certain physiological peculiarities which yellow mice exhibit to a greater extent and in a greater degree than do non-yellows. Among these characteristics may be mentioned a marked tendency to adiposity. Danforth (1927) has published an account of this condition and has shown that albino mice which possess the factor for yellow A^y become adipose (as do actual yellows) even though they form no pigment. This suggests, therefore, that mice with the gene A^y usually have a peculiar metabolism. To what extent the type of metabolism ordinarily found in yellow mice is restricted to mice possessing the gene (A^y) for yellow is uncertain. Adult weights in grams of 38 F_1 virgin female non-yellows and 41 virgin F_1 female yellows show a distinctly higher average weight for the yellows with a distribution that is clearly different for the two color types. There is some overlapping. What effect, if any, adiposity may have on the incidence of mammary tumors is worthy of further study.

It is also true that yellow mice frequently show, in comparison with non-yellows of the same stock, an altered reproductive cycle and an increased incidence of sterility. Exact data, however, on all of these metabolic differences still remain to be gathered.

In this connection it is important to note the comparative age of tumor incidence in yellows and non-yellows. No special effort has been made to establish in either case an exact date to which the incidence of cancer may be referred. To make such an effort and to place on its outcome too great reliance would be misleading. In the experiment here reported all mice with mammary tumors were killed when a clearly palpable tumor nodule was present. This situation is

reached at strikingly different mean ages in yellows and non-yellows. The mean age for 22 F_1 yellows is 401 days, for 33 F_1 non-yellows 521 days. The mean age of 58 yellow F_2 mammary tumor mice is 482 days while that of 115 non-yellow mammary tumor mice is 564 days. The difference of 82 days is certainly suggestive of the fact that yellow mice reach tumor age and develop mammary tumors *earlier* than do non-yellows of the same generation. This holds for both the F_1 and F_2 generations. The fact that yellow mice may develop mammary tumors earlier although significantly less frequently than non-yellows has a general bearing on the findings of Loeb and other early investigators who observed that cancer incidence and cancer age were to some degree at least independent of one another. Loeb (1921) has, however, stated that as a general thing strains which have a high incidence of mammary tumors tend also to have an earlier average age incidence of these tumors than do the low tumor strains. In the material included in the present communication there is an exception to what Loeb believes to be the general relationship between tumor age and tumor incidence.

It is also interesting to record roughly the degree of malignancy in the mammary tumors of yellow and non-yellow mice in the F_1 and F_2 generations. This is done in Table XVIII.

From Table XVIII it will be seen that there is no evidence that yellow mice develop less malignant types of mammary tumors than do non-yellows. The evidence is in fact somewhat in the other direction.

Thus although yellow mice form significantly fewer mammary tumors than do the non-yellows of similar generations, those that form tumors do so earlier and give rise to, if anything, more malignant neoplasms than do the non-yellow animals.

There has been, therefore, a decrease in cancer incidence among yellow mice, but no signs of a weakening of the actual process of cancer formation itself.

The most probable explanation for the observed facts would seem to be found in the general physiology of the yellow animals. We know certain things about yellow mice of this stock. In both F_1 and F_2 generations yellow non-tumor mice die at an average earlier age than do non-yellows. The differences are shown in Table XIX. In each case the yellows die at a distinctly earlier age than do the non-yellows.

TABLE XVIII

		No.	Per cent
F₁ yellow:	Total mammary tumors		
	Adenoma	22	
	Carcinomatous	3	13.6
	Mixed (transition)	16	72.7
		3	13.6
F₂ yellow:	Total mammary tumors		
	Adenoma	58	
	Carcinomatous	11	19.0
	Mixed (transition)	44	75.9
		3	5.1
Total yellow:			
	Adenoma	80	
	Carcinomatous	14	17.5
	Mixed (transition)	60	75.0
		6	7.5
F₁ non-yellow:	Total mammary tumors		
	Adenoma	35	
	Carcinomatous	5	14.3
	Mixed (transition)	19	54.3
		11	31.4
F₂ non-yellow:	Total mammary tumors		
	Adenoma	115	
	Carcinomatous	36	31.3
	Mixed (transition)	77	66.9
		2	1.7
Total non-yellow:			
	Adenoma	150	
	Carcinomatous	41	27.3
	Mixed (transition)	96	64.0
		13	8.7

TABLE XIX

Non-tumor mice	Mean age at death
	days
F₁ yellow	632.0
non-yellow	813.0
F₂ yellow	622.0
non-yellow	660.0
Combined F₁ and F₂ yellow	624.0
non-yellow	690.0

Although the total number of animals in each case is not large the difference is about 3.2 times its probable error. This coupled with its consistency in both generations suggests very definitely that there is a significant difference in length of life between yellow, and non-yellow, non-tumor-bearing animals. This considered along with the fact that yellows form their mammary tumors at a distinctly earlier age, tends to support the idea that yellow mice pass through the periods of life preceding senility at a more rapid rate than do their non-yellow relatives. This would account for the observed difference in mean age of incidence of mammary tumors in yellow and non-yellow mice. It would also explain the shorter life span of yellows in that we might expect the total duration of life to show the effect of the premature aging of the yellows during their reproductive cycle. A more rapid aging of this type would also give the impression of greater sterility. Yellow mice, in breeding pens with non-yellows would, by hypothesis, reach the end of their reproductive cycle at an earlier absolute age than that of the non-yellows.

The complete breeding cycles of 25 non-yellow females whose mates were yellow males may be compared with those of 31 yellow females mated with non-yellow males. In each case both parents are taken from the inbred yellow stock used as one of the races which were crossed together in this experiment.

The yellow females produced a total of 396 young of which 104 or 26.2 per cent died before weaning. The non-yellow females gave a total of 350 young of which 24.0 per cent died before weaning. Lactation in the yellows and non-yellows therefore appears to be approximately equally effective. The yellow females produced on the average 2.5 litters apiece, the non-yellows 2.7. This is a slight excess of reproductive activity on the part of the non-yellows. There is another fact that bears upon the relative reproductive activity of the two types. The average span of parturitive activity in the non-yellows is 117 days and in the yellows 105 days. We may conclude, therefore, that in general the absolute duration and intensity of parturitive activity is essentially similar in the two groups although non-yellows have a slight advantage.

The mean litter size for yellow females is 5.01 young and for non-yellows 5.15 young. This tends to show, along with the average num-

ber of litters which each type has, that they are similar. The numbers available are not sufficiently large to determine whether the slight but consistent inferiority of the yellows in each of the four criteria is or is not a significant physiological difference between them and non-yellows of the same stock.

There remain certain matters bearing on the relative rate of maturity and the onset of senility which should be considered.

In this connection marked differences begin to show between the two groups. The average age of yellows at the first litter is 116 days while that of non-yellows is 154 days. This is a difference of 38 days. While larger numbers might well change the actual values it is extremely doubtful whether they would obliterate the difference between the two types. Similarly the mean age at which yellows have their last litter is strikingly earlier than that of the non-yellows, the figures are 222 and 271 days respectively. The physiological relationship of the mean age at first litter, mean age at last litter and, in the hybrids, mean age of death and mean age of incidence of mammary tumors is therefore a consistent one. If in each case the value for yellows is taken as 100 the relation to non-yellows can be compared. When this is done it is seen that in every case the value for non-yellows is higher than that of yellows.

There remains to be explained why the incidence of mammary tumors should be significantly less in yellows than in non-yellows. The probable reason for this is to be found in the fact that in the non-yellows there is a longer period for the secretions of the ovary or other parts of the endocrine system involved in reproductive activity to affect the mammary tissue of tumor-producing age than there is in the yellows. The work of Murray has clearly shown that continued influence of ovarian secretion plays an important rôle in the occurrence of mammary tumors in the dilute brown strain of mice. There is no reason to believe that the conditions described by him are not typical for all mice. The need of continued irritation by tar is also recognized by those who use that substance to induce the incidence of epithelial growths in mice.

In yellow and non-yellow mice of this stock the mammary system is approximately equal in efficiency. The possibility certainly exists that the ovary of the yellow mouse passes through its span of activity

so rapidly in relation to that of the non-yellows that the period during which endocrine secretions irritate the mammary tissue, is, in the former, earlier in the life of the mouse and shorter in duration. These two factors would contribute towards making yellow mice less apt to produce mammary tumors than are non-yellows.

This explanation may serve as a working hypothesis until further research on the subject is reported.

Work is now being planned to test whether it is necessary for the ovary to age or whether continued stimulation from secretions of young ovaries will produce the same result. The simpler hypothesis is that continued irritation of mammary tissue by any complete ovarian secretion will suffice to bring to expression any tendency to form mammary tumors wherever such a tendency exists.

The Relation of Dilute Pigmentation to Tumor Growth

Since all F_1 generation animals carry dilution d^b as a recessive, the F_2 generation provides the first real test as to whether the presence of dilute pigmentation has any noticeable effect on tumor incidence.

Table XVII shows that the percentage of mammary tumors in the four F_2 color classes is as follows:

	per cent
Yellows	42.6
Dilute yellows	25.0
Browns	53.8
Dilute browns	46.3

In both yellows and non-yellows the dilute animals show a lower incidence of mammary tumors than does the corresponding color class with intense pigmentation. This may not be significant although the total dilutes in showing 37.5 per cent mammary tumors depart from the total intense (49.2 per cent) by 3.4 times the probable error of the difference. At the same time the dilute animals show actually a slight excess of tumors other than mammary—when compared with the intense mice (10.4 and 7.2 per cent respectively).

The dilute yellow group which combines both the color types unfavorable to mammary tumor incidence shows distinctly the lowest percentage of such tumors. This would be expected if the two color types—yellow and dilute—were independently unfavorable in their physiological effect on mammary tumor incidence.

Further investigations and larger numbers of young are needed before any detailed conclusions as to the nature of the effect of dilution can be drawn.

SUMMARY AND CONCLUSIONS

1. The material included in this paper consists of F_1 and F_2 virgin female mice derived from a cross between a strain high in mammary cancer incidence (dilute brown) and one relatively low in incidence of mammary cancer but relatively high in the incidence of various internal tumors (yellow).

2. In the F_1 and F_2 hybrid generations the yellow animals have a significantly lower incidence of mammary tumors than do the non-yellows. This is the first clear case of a difference in the incidence of spontaneous tumors in mice associated with a color difference.

3. Mammary tumors occur, however, significantly earlier in the yellow mice and are just as malignant as those appearing in the non-yellows.

4. The incidence of tumors other than mammary is not significantly different in the yellow and non-yellow hybrids. Such tumors, however, occur distinctly later in life than do the mammary tumors. This provides additional evidence that, in mice, mammary tumors cannot be considered to be the same biological phenomenon as are other types of tumor.

5. A study of the physiology of reproduction of yellow and non-yellow mice within the yellow stock suggests that the yellows pass through their reproductive cycle earlier than do the non-yellows. The *duration* of the cycle in the two forms is essentially equal. This fact would satisfactorily explain the earlier incidence of mammary tumors in yellow mice.

6. The lower incidence of mammary tumors in yellows as compared with non-yellows may be at least in part due to the same phenomenon. This would follow because the opportunity for mammary tissue in yellow mice of cancer age to be continuously affected by ovarian secretion would be less than in non-yellows. This would result in a higher percentage of yellows reaching an age at which stimuli from the ovary ceased before the mammary tissue had reached an age at which tumor formation is most frequent.

7. There is some evidence that, in this cross, dilute ($d^b d^b$) mice are less apt to form mammary tumors than are intensely pigmented animals. This point, however, needs further investigation before it can be considered to be established.

8. The facts recorded in this paper demonstrate that not all forms of tumor or all colors of mice can be lumped together in studying either the physiology or genetics of spontaneous tumor incidence.

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BLOOD PLASMA PROTEIN REGENERATION CONTROLLED BY DIET

I. Liver and Casein as Potent Diet Factors

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We have ample evidence to show that blood plasma protein regeneration in the dog can be modified at will by diet factors. The evidence in this paper indicates that liver and casein feeding are very potent in effecting rapid plasma protein regeneration. Vegetable diets may be less effective as shown below and reported in the publications from other laboratories. Possibly other proteins in the diet may prove to be even more efficient than liver in bringing about plasma protein regeneration. We are testing systematically other proteins. No one can group proteins into complete or incomplete, vegetable or animal, and by that grouping indicate the value of any protein for the regeneration of plasma protein. Individual proteins must be tested in the fire of metabolism. It will be of some interest to determine in what protein fractions or split products this potency may reside.

Eventually all this information will be of interest to the physician who deals with human cases showing hypoproteinemia or blood protein loss. The maintenance of blood proteins above the edema level has a practical bearing in the treatment of certain human cases of nephrosis and nephritis.

The general experimental plan is extremely simple but a certain number of technical difficulties had to be overcome. Dogs are reduced to a blood plasma protein level of approximately 4.0 per cent just above the edema level (3.3–3.8 per cent) and held continuously at this level by suitable bleedings combined with replacement of red cells suspended in Locke's solution (plasmapheresis). This low plasma protein level acts as a stimulus for the production of new plasma pro-

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teins presumably in maximal amounts. It was soon found that a normal dog has a considerable amount of material stored in the body out of which plasma proteins can be fabricated. The same thing (23) is true for hemoglobin in the dog. When this reserve is exhausted with the dog on a basal diet the animal can still produce 15–30 gm. of new plasma protein each week. The basal diet is poor in vegetable protein and contains no animal protein, yet the dog can be kept in nitrogen equilibrium with actual gain in weight if the diet is eaten over periods of many months (Dog 32-58).

When the basal diet is replaced by cooked liver or supplemented by casein we note a large output of new plasma protein, several times the basal diet output (Table 3). The excess output due to liver or casein feeding for 1 week may amount to 60–70 gm. plasma protein over and above the basal output.

Space will not permit a complete review of earlier work in this field. Bloomfield (3) has recently reviewed much of the literature dealing with edema and blood plasma protein concentration. He points out that the "loss and lack theory" does not seem adequate to explain all the observations on serum proteins. We believe that the *reserve store* of protein or protein building material shown in the tables below will clear up some of the discrepancies in the literature. The specific action of various dietary proteins will help still more in the solution as will the observations in the second paper which indicate that *plasma proteins may be utilized in the body economy*. We do not refer to the many papers reviewed by Bloomfield but list a few of the important ones in the bibliography.

Henriques and Klausen (7) using rabbits with phosphorous poisoning and dogs with bile duct ligation record a decrease in plasma albumin but not in globulin. Edema developed in some instances. They claim this is proof that albumin is formed in the liver and globulin elsewhere. They did not control the diet. Compare Dog 32-394, Table 1 below, where the same change is noted as due to many weeks of the "basal ration" and nothing else.

All investigations by means of plasmapheresis have been done with the dog. Kerr, Hurwitz, and Whipple (10) first pointed out the influence of diet factors on plasma protein regeneration after plasmapheresis. The dog regenerated these proteins much faster on a mixed diet than during a fasting period. There was evidence of an emergency *reserve store of proteins*. Liver injury impaired this reaction and indicated that the liver might be concerned with maintenance of the blood plasma protein concentration. Whipple, Belt, and Smith (22) confirmed and extended the above observations. The liver was believed to be concerned with plasma protein regeneration. Barker and Kirk (1) were particularly interested in edema produced in the dog by plasmapheresis. The diet used was not

given but must have been rich in protein. Leiter (13) was interested especially in edema produced by plasmapheresis. A bread and meat diet of unknown amount was used. Shelburne and Egloff (18) used a limited diet of potatoes, cream, turnips, butter, and lactose in dogs with and without plasmapheresis. They note a change in the albumin-globulin ratio and fall in total protein. Edema was readily produced by an administration of saline by stomach tube after 83 days on this diet. Barnett, Jones, and Cohn (2) showed that on a liberal meat diet the dog could regenerate large amounts of plasma protein removed by plasmapheresis. They doubt the emergency storage of protein. Darrow, Hopper, and Cary (4) made similar observations. Weech (19, 20) and his collaborators used dogs to study the effect of a low protein diet alone and with plasmapheresis. They noted a fall in albumin and a rise in globulin as edema developed. They studied the nitrogen balance and gave small amounts of serum intravenously to combat the edema without result. Lepore (14) using a milk and bread diet combined with plasmapheresis in dogs was able to reduce the total plasma proteins to 3.5 per cent, and observed edema when fluids were forced.

Important tests with human patients are reported by Liu, Chu, Wang, and Chung (15). They studied the effect of different levels of animal and vegetable protein intake on nitrogen balance, plasma proteins, and edema in two cases of nutritional edema. They believe animal protein is twice as effective as vegetable protein for plasma protein regeneration.

Methods

Dogs were used in all experiments and the basal ration was fed usually some days or weeks to make sure that the animal would take it freely. Dogs were protected against distemper with the Laidlaw-Duncan vaccine and unless otherwise noted were in normal health.

The *basal ration* is a result of our experience and that of others. It contains no animal and but little vegetable protein—about 7 per cent of its caloric value being protein. This ration does not appeal to all dogs but many will consume adequate quantities over months and remain in nitrogen equilibrium with even some gain in weight. The basal ration consists by weight of 40 parts boiled white potatoes, 20 parts canned tomatoes, 10 parts Post bran flakes, 10 parts karo corn syrup, and 5 parts cod liver oil. Each dog received daily, with this diet, 1 gm. of the McCollum-Simmonds salt mixture (16). The weighed basal ration was placed in the cage in the afternoon and the amount consumed recorded in the tables. Water was available in the cages at all times.

Plasmapheresis in these experiments means the removal of 25–40 per cent of the blood volume as estimated by the dye method (Hooper, Belt, Smith, and Whipple (8)). Immediately following the bleeding and sometimes during the last part of the bleeding there is injected intravenously a mixture of normal red cells suspended in Locke's solution. The red cells for injection were obtained from

healthy donors. For this purpose large dogs were used and the amount of bleeding was regulated so that they should show no anemia. A donor was bled 300–400 cc. in the morning and the red cells separated from the plasma by centrifugalization—3,000 R.P.M. for 35 minutes in 100 cc. tubes. The plasma was removed by suction, the cells washed in modified Locke's solution and recentrifugalized. The constituents of the Locke's solution were mixed each morning, the calcium salt being left out because of its tendency to cause coagulation. The washed red cells were then resuspended in Locke's solution containing 5 per cent glucose and heated to 37°C. for injection. The volume was maintained at about 10 per cent more than that originally bled. It was found that this single washing as described removed more than 99 per cent of the plasma protein. The blood was usually removed from the femoral artery and the washed red cells injected into the jugular vein by means of a gravity bottle. 1 cc. of concentrated Na citrate per 50 cc. of blood was used as an anticoagulant. The dilution of the plasma due to the citrate was corrected for in calculating the total protein. Daily hematocrits were taken, using 2 cc. of 1.4 per cent sodium oxalate, and these are recorded as an indication of the dog's hemoglobin level. An excess of red cells was always injected as the trauma of the centrifugalization injures many of the introduced red cells and they disintegrate with the appearance of some hemoglobin in the blood plasma.

During the initial period and liver or casein periods it is necessary to bleed and replace maximal amounts to reduce the plasma protein levels. Five or six bleedings a week are necessary. It was thought best not to attempt to maintain the red cell hematocrit at 50 per cent, or normal, but Dog 32-30 was maintained at approximately 30 per cent at which anemia level dogs will show no clinical disturbance. Moreover the gross bleeding required will be less to remove a given amount of plasma protein.

The blood from the dog on basal diet was centrifugalized at 3,000 R.P.M. for 35 minutes and the plasma drawn off as completely as possible by suction. All analyses were run on this sample. The total volume of plasma removed was accurately measured and checked against the total amount bled and the hematocrit. By allowing 1 cc. for each centrifuge tube used as the amount of plasma that could not be drawn off without contaminating the plasma with red cells, it was possible to measure amounts that checked within 2 per cent of the calculated yield. Since the single most important figure in these studies is the *total grams of plasma protein removed*, particular attention has been paid to all factors which might alter this figure. It is recognized that injecting washed donor's corpuscles before completion of bleeding, by dilution, lowers the protein concentration of the plasma removed, that during a large bleeding some dilution may normally occur in an attempt to restore the blood volume, and that the use of hypertonic citrate certainly dilutes the plasma by taking fluid out of the red cells. For these reasons too much emphasis is not placed on the column listed "Blood plasma average concentration." But none of these factors alter the actual number of grams of

plasma protein removed, since this is figured as the product of the cubic centimeters of plasma removed and the concentration of that removed. And since any dilution is accomplished by fluid that is presumably very nearly protein-free, it is believed that the figures for the fractionation into albumin and globulin are absolute within the limits of error of the method.

This citrated blood was centrifugalized and the plasma accurately measured. The *plasma* was then analysed for total nitrogen by the macro-Kjeldahl method, using 1 cc. of plasma. Non-protein nitrogen was determined by duplicate macro-Kjeldahl analyses of 50 cc. of Folin-Wu filtrate, 200 cc. of which were prepared with 20 cc. of plasma. The analysis of albumin and globulin was done according to Howe's method as described by Peters and Van Slyke (17) using 22 per cent sodium sulfate at 37°C.; triplicate analyses were carried out. The dogs were kept in clean metabolism cages during the entire experiment. The weekly *urinary output* was collected in large bottles, using 20 cc. of concentrated H_2SO_4 as a preservative. The volume was measured and the urine analysed for total nitrogen, using 1 cc. aliquots with the macro-Kjeldahl procedure. Thus the macro-Kjeldahl method was employed in all analyses and in all determinations duplicate or triplicate analyses were made and repeated if they did not check to within 1 part in 60. This reduces the probable error to less than 1 per cent.

Fecal nitrogen was not determined routinely but in many experiments the fecal nitrogen was calculated for 1–2 weeks and this figure used throughout the experiment. Obviously the nitrogen balance is not absolutely accurate but the error probably is not large. In some experiments (Dog 32-30) the fecal nitrogen was figured as 1 gm. per day and not analysed. The figure is too high and increases the negative balance as given.

Total protein removed as given in the table is probably about 5 per cent too low as the washings from the centrifugalized red cells contain about this amount of protein and this correction is not made. However the error is relatively constant and makes the reaction to diet factors a little less conspicuous than would the corrected figures.

EXPERIMENTAL OBSERVATIONS

The first thing to determine was the behavior of a normal dog on the basal ration. Some dogs will not eat this diet in sufficient amount to maintain weight.

Basal Ration and Normal Dog

Dog 32-58.—A small adult female mongrel; received the basal diet 425 gm. per day plus 1 gm. salt mixture, the composition as described under Methods. This amounted to about 100 calories per kilo body weight. The dog ate 75 to 100 per cent of this diet every day for 27 weeks while under observation, and was in positive nitrogen balance. The final weight was 7.1 kg. as compared with 5.8 kg.

at the start. The dog was in perfect health throughout. The plasma proteins were followed at intervals during this long period. The control level was unusually high—7.8 per cent total protein. After 1 week on the basal diet this high value fell to 6.1 per cent total protein in blood plasma. For the next 10 weeks there

TABLE 1

Blood Plasma Protein Depletion and Regeneration
Initial Reserve of Normal Dog

Periods 7 days	Diet	Total protein removed	Estimated basal output	Total protein removed above basal			Blood plasma Average concentration		
				Total protein	Albumin	Globulin	Total protein	Albumin	Globulin
Dog 32-394. Basal ration for 2 mos. before beginning depletion									
		gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
1	Basal	44.7	25	19.7	7.5	12.2	4.82	1.76	3.05
2	"	30.5	25	5.5	—	—	3.80	—	—
3	"	28.9	25	3.9	—	—	3.57	—	—
4	"	25.0	25	0.0	0.0	0.0	3.30	—	—
Total reserve above basal.............				29.1					
Dog 32-168. Basal ration for 1 wk. before beginning depletion									
Control							4.80	2.81	1.99
1	Basal	85.8	35	50.8	—	—	4.09	—	—
2	"	69.0	35	34.0	17.2	16.8	3.75	1.89	1.86
3	"	57.9	35	22.9	13.2	9.7	3.35	1.51	1.84
4	"	32.7	—	—	—	—	3.72	—	—
Total reserve above basal.............				107.7					
Dog 32-290. Basal ration for 1 wk. before beginning depletion									
Control							6.62	3.53	3.09
1	Basal	86.7	30	56.7	36.7	20.0	5.42	3.44	1.98
2	"	69.5	30	39.5	23.1	16.4	4.61	2.69	1.92
3	"	55.2	30	25.2	12.3	12.9	4.01	1.97	2.04
Total reserve above basal.............				121.4	72.1	49.3			

was no change in the level of plasma protein. There was no change in the red cell hematocrit at any time. During the 16th and 17th week the total blood plasma protein is recorded as 4.9 per cent and 4.7 per cent. In the 26th and 27th weeks the plasma protein values are recorded as 4.7 and 5.2 per cent. Evidently on this

basal diet this dog could maintain a protein concentration in its blood within normal limits.   On several occasions the albumin-globulin ratio was determined and found to be normal.   This is in contrast to Dog 32-394, Table 1, where there is a reversal of the albumin-globulin ratio after 2 months' feeding of the basal ration.

Table 1 shows the amounts of plasma protein which must be removed in normal dogs to exhaust the *reserve storage* of plasma proteins or their

TABLE 2

Bleeding, Nitrogen Balance, and Clinical Condition

Periods 7 days	Food consumption	Weight	Edema	Negative N balance	Total bleeding	R.B.C., hematocrit
Dog 32-394. Basal ration 525 gm. daily						
	per cent	*kg.*		*gm.*	*cc.*	*per cent*
Control	100	13.4	0	—	1,500	36.6
1	100	13.7	0	—	1,255	39.7
2	100	13.8	0	—	1,400	41.8
3	100	13.7	0	—	1,120	37.6
Dog 32-168. Basal ration 850 gm. daily						
Control	100	21.6				
1	55	21.5	0	20.0	3,300	33.8
2	43	21.0	0	35.4	2,875	30.5
3	33	20.5	+++	24.0	2,575	25.2
4		20.0	+++	13.3	1,325	20.5
Dog 32-290. Basal ration 420 gm. daily						
Control	100	16.6				
1	100	15.2	0	35.4	2,560	34.6
2	95	14.5	+	44.9	2,110	24.0
3	67	13.3	0	41.4	1,970	30.1

parent substances.   Compare Table 3 below where the periods of observation are longer and the basal level more convincing because determined on two different occasions after long periods of plasmapheresis.

The basal output per week on this diet is close to 2 gm. plasma protein per kilo per week.   There is a tendency for this value to fluctuate a little as the experiments run on for several months (Table 3) but the

amount seems reasonably constant under the conditions of these experiments.

The *reserve storage* amounts to quite a respectable figure but appears to be lower in dogs which have been on the basal diet for some time (Dog 32-394). There may be other factors which determine the amount of this reserve storage but in these dogs it varies from 30 to 120 gm. total plasma protein—2 to 7 gm. per kilo body weight (Table 1—Total protein removed above basal).

Albumin appears as a conspicuous feature in this reserve store and usually makes up one-half or more of the total protein removed. This is in conspicuous contrast to the protein removed subsequently where globulin makes up more than twice the amount of albumin removed (Table 3).

Clinical History, Dog 32-394.—An adult female bull mongrel weighing 15.0 kg. This dog had been on the basal ration for 2 months. The plasma protein level following this basal period was 4.82 per cent, albumin 1.76 per cent, and globulin 3.05 per cent. The blood volume was 920 cc., the plasma volume 560 cc. Throughout the periods of observation here recorded the dog was in excellent condition. This dog is being used for diet experiments and plasma regeneration at the present time (November, 1933) and is in excellent condition.

Clinical History, Dog 32-168.—An adult shepherd mongrel male weighing 21.8 kg. The initial level of plasma protein was 4.8 per cent, albumin 2.81 per cent, and globulin 1.99 per cent. The blood volume was 1,600 cc., the plasma volume 760 cc. Plasmapheresis was done with exchanges of 500 cc. of blood. At the end of the 4th week there was difficulty in entering the jugular vein to inject the washed cells following a large bleeding. The dog went into shock and died in 3 hours. Autopsy showed viscera normal but for anemia.

Clinical History, Dog 32-290.—An adult female terrier whose normal weight was 16.5 kg. The initial plasma protein level was 6.62 per cent, albumin 3.53 per cent, and globulin 3.09 per cent. The blood volume was 1,283 cc., the plasma volume 690 cc. At the beginning exchanges of 450 cc. were performed. At the beginning of the 2nd week the dog started vomiting and developed hemoglobinuria. Subsequent exchanges did not exceed 350 cc. of blood. Pitting edema developed during the 3rd week and persisted during the dog's life. The dog was found dead in the cage at the end of the 3rd week following an exchange of 350 cc. on the preceding day. Autopsy showed bronchopneumonia, pleural effusion left, acute tricuspid and mitral endocarditis.

Table 2 gives more data related to the experiments outlined in Table 1. Dog 32-394 is of particular interest because it is obvious that a dog eating all the basal ration can maintain its weight during a period

of plasma depletion. This dog showed no edema at any time in spite of a rather low plasma protein level. The red cell hematocrit in this dog was held close to 40 per cent, a low normal figure.

Dog 32-168 is a satisfactory experiment in which the dog did not eat the basal ration well and lost weight. Edema developed in the last 2 weeks and the hematocrit was too low: this perhaps helps to explain the edema appearing at plasma protein levels of 3.7 per cent. The dog died as the result of an accident and all viscera were normal at autopsy.

Dog 32-290 is the least satisfactory of these three experiments in Table 2. This dog in the last week or two of life developed an acute infection of the lungs and endocardium. There was conspicuous loss of weight in spite of a good food consumption. This unusually high output of plasma protein may be due in part to infection which is known to stimulate a great overproduction of fibrinogen. It is significant however that the albumin remained high in all this reserve output.

The Effects of Liver or Casein Feeding

Table 3 shows a successful experiment lasting 19 weeks in which the output of blood plasma proteins due to *liver* or *casein* feeding is in conspicuous contrast with the reaction to the basal diet. This surplus production due to either liver or casein is about 70 gm. plasma protein per week over and above the basal level while the reaction to the basal diet is about 20 gm. plasma protein per week.

The ratio of food protein intake to plasma protein production is of considerable interest. With liver feeding the total liver protein intake amounted to 893 gm. while the plasma protein regeneration above the basal level for the 2 week period is recorded as 131 gm. Each 6.8 gm. of liver protein was responsible for the production of 1 gm. of plasma protein surplus. The reaction to casein is not quite as striking but of the same order. Approximately 700 gm. of commercial casein as food intake were responsible for a total plasma protein surplus of 70 gm. (Table 3) or a ratio of 10 gm. casein to 1 gm. of produced plasma protein. It is to be kept in mind that the *liver* ration *replaced* the basal ration while the *casein* was *added to* the basal ration. A more accurate experiment would show the *addition* of the liver to the basal ration.

TABLE 3

Blood Plasma Protein Depletion and Regeneration
Liver and Casein Compared with Basal Diet

Dog 32-30.

Periods 7 days	Diet	Total protein removed	Total protein removed above basal*			Blood plasma Average concentration			N.P.N.
			Total protein	Albumin	Globulin	Total protein	Albumin	Globulin	
		gm.	gm.	gm.	gm.	per cent	per cent	per cent	mg. per 100 cc.
1	Low	39.6	19.6	10.1	9.5	4.08	2.09	1.99	17
2	Basal	30.1	10.1	5.3	4.8	4.00	2.11	1.89	20
3	"	37.2	17.2	9.0	8.2	3.38	1.76	1.62	18
4	"	34.3	14.3	7.0	7.3	3.65	1.79	1.86	20
Total reserve above basal*..............			61.2	31.4	29.8				
5	Basal	3.6		1.3	2.3	4.11	1.50	2.61	16
6	"	28.1		9.8	18.3	3.60	1.26	2.34	19
7	"	10.8		4.0	6.8	2.98	1.14	1.84	16
Average basal*..........		14.1		5.0	9.1				
8	Liver	27.7	27.7	11.7	16.0	4.61	1.94	2.67	23
9	"	53.5	53.5	26.4	27.1	4.61	2.27	2.34	35
10	Basal	27.0	7.0	3.0	4.0	4.62	1.97	2.65	20
11	"	42.4	22.4	7.9	14.5	4.23	1.49	2.74	18
12	"	31.0	11.0	3.7	7.3	4.06	1.37	2.69	19
13	"	30.0	10.0	3.2	6.8	3.79	1.22	2.57	20
Total regeneration due to liver..........			131.6	55.9	75.7				
14	Basal	25.4		8.1	17.3	3.48	1.10	2.38	17
15	"	16.6		4.9	11.7	4.19	1.23	2.96	12
16	"	21.1		7.1	14.0	3.46	1.15	2.31	15
Average basal*..........		21.0		6.6	14.4				
17	Casein	53.0	33.0	13.9	19.1	5.24	2.21	3.03	38
18	Basal	48.1	28.1	11.0	17.0	5.02	1.81	3.21	20
19	"	29.0	9.0	3.9	5.1	3.78	1.64	2.14	20
Total regeneration due to casein........		70.1	28.9	41.2					

* Estimated basal output equivalent to 20 gm. plasma protein per week.

The ratio of food protein (vegetable) in the basal diet to plasma protein production is 88 gm. food protein per week and 20 gm. basal output per week—or 4.4 gm. vegetable protein are responsible for the production of 1 gm. plasma protein. Obviously this vegetable protein is utilized to very good advantage by the dog under these conditions

TABLE 4

Bleeding, Nitrogen Balance, and Clinical Condition

Dog 32-30.

Periods 7 days	Diet	Food consumption	Weight	Edema	Negative N balance	Total bleeding	R.B.C., hematocrit
		per cent	*kg.*		*gm.*	*cc.*	*per cent*
1	Low	80	9.3	0	21.2	1,615	33.9
2	Basal	89	9.2	0	20.2	1,290	28.3
3	"	50	9.6	0	20.4	1,905	28.1
4	"	50	9.9	+++	16.9	1,460	26.3
5	"	88	9.5	++	9.1	155	29.1
6	"	73	7.7	0	11.6	1,220	29.4
7	"	78	8.8	+++	8.2	575	34.8
8	Liver	100	8.2	0	+94.2	990	37.7
9	"	100	8.5	0	+82.5	1,925	35.9
10	Basal	95	8.9	0	0.7	935	37.1
11	"	100	8.8	0	13.6	1,440	30.0
12	"	98	8.7	0	11.4	1,240	38.1
13	"	98	8.6	++	9.4	1,190	31.1
14	"	97	8.6	+++	9.1	1,105	37.2
15	"	100	9.1	++	5.3	620	35.7
16	"	91	8.3	++	9.2	950	34.0
17	Casein	90	8.7	0	+51.8	1,500	29.1
18	Basal	58	8.3	0	36.4	1,500	32.8
19	"	75	7.8	0	14.6	1,220	26.1
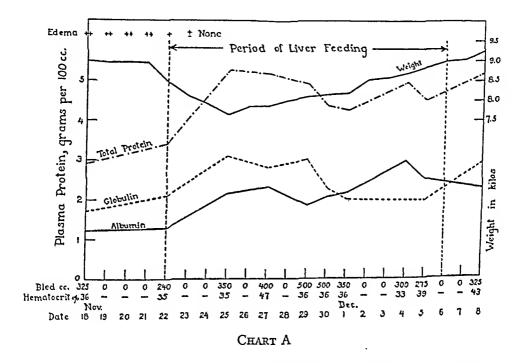
Basal ration 425 gm. daily.

and we cannot say that the liver protein per 100 gm. ingested is more or less efficiently utilized. It is possible that the liver protein if given in such small amounts would be as completely utilized. Nor can we support the thesis of the Chinese investigators (15) that animal protein is worth twice as much as vegetable protein. Much more work is needed and from what is known at present we can only say that each

protein must be tested in the animal economy to give the answer to this question.

The *reserve storage* (Table 3) amounts to 61 gm. plasma protein over and above the basal production on the standard basal diet—or 6.7 gm. per kilo body weight. It is of interest that albumin and globulin in about equal amounts (31.4 and 29.8 gm.) are represented in this reserve store.

The *basal output* per week (5th to 7th weeks) averages 14.1 gm. plasma protein but at a later period (14th to 16th weeks) averages 21 gm. plasma protein. During both these periods the globulin removed amounts to twice the amount of albumin. Evidently on this basal

CHART A

ration the dog can form twice as much globulin as albumin. It is possible that the vegetable and grain proteins favor the formation of globulin more than do the animal proteins.

Liver and casein diet periods show a greater amount of albumin production and during the 2nd week of liver feeding the albumin output practically equals the globulin production (Table 3 and Chart A).

It is to be noted that this Dog 32-30 with a blood plasma volume of approximately 450 cc. had in circulation normally about 23 gm. total

plasma protein.   The production per week on the basal ration (20 gm. plasma protein) is almost as much as the circulating proteins while on the liver diet this figure per week may be 65 gm. plasma protein total output.   The reserve of 61 gm. amounts to roughly three times the normal amount of plasma protein in circulation.

Clinical History, Dog 32-30.—A young adult female about 1 year of age, initial weight 10.1 kg.   The plasma protein was 5.2 per cent, albumin 3.3 per cent, and globulin 1.9 per cent.   The blood plasma volume during the long period of observation (5 months) varied but little and averaged about 450 cc.   During the edema periods the blood plasma volume showed only slight change and averaged about 420 cc.   The 1st week of the experiment the dog was on a low protein diet in which rice or white bread supplied the only protein.   After this for the remaining 19 weeks the dog was on the basal ration used in the other experiments.   The food ration was given as described 425 gm. per day and the percentage consumption indicated in Table 4.   The 1st day's bleeding amounted to 750 cc. whole blood in three separate exchanges done under ether anesthesia by placing cannulas in femoral vein and artery (September 30, 1932) (22).   Following this the dog was bled by needle puncture of jugular vein or femoral artery.   Red blood cells suspended in glucose Locke's solution were injected into jugular or femoral veins. From December 15, 1932 to February 21, 1933, blood was removed by cardiac puncture.   On many occasions after several days of bleeding quite marked hemolysis followed by icterus would be noted.   Coincident with this hemolysis, diarrhea and vomiting might ensue and the dog would refuse a part of its diet. Bleeding would be discontinued for a day or two, the clinical condition would become normal, and the food intake return to the usual level.

The *liver diet period* of 2 weeks means a diet of cooked whole pig liver 300 gm. (fresh weight) daily.

The casein diet period means 100 gm. commercial casein per day *added to the basal ration* of 425 gm. per day.

During the last week of life the dog showed signs of infection with fever, icterus, prostration, and loss of appetite.   This week was not included in Tables 3 and 4. Autopsy—Bilateral bronchopneumonia and acute pleuritis.   Acute mitral and aortic and subacute tricuspid endocarditis (cardiac punctures).

Table 4 gives other experimental data on Dog 32-30 and supplements Table 3.   It is seen that the food consumption is excellent but there is a slight loss of weight.   There is a continuous negative nitrogen balance but the figures are somewhat high as the fecal nitrogen was not analysed but figured as 1 gm. per day.   The plasma nitrogen removed by plasmapheresis is included in the nitrogen balance.   The conspicuous positive nitrogen balance during liver and casein feeding is well

shown. The red cell hematocrit is too low to be considered normal but it is known from anemia work with dogs in this laboratory that this degree of anemia does not cause any recognizable clinical or physiological disturbances.

Edema appears when the total protein level approaches 3.5 per cent whether the albumin-globulin ratio is 1/1 or 1/2. The total protein level would seem more important than the albumin-globulin ratio or the total albumin concentration. Ascitic fluid obtained on one occasion showed 0.93 per cent total protein of which 0.26 per cent was albumin and 0.67 per cent globulin.

Chart A illustrates beautifully the reaction of Dog 32-30 on the basal ration to a diet of liver. The dog showed a very low plasma protein level (plasmapheresis) with tissue edema and ascites. At first the albumin-globulin ratio is reversed but after a week of liver diet the albumin fraction returns toward normal and even shows the normal preponderance of albumin. As the edema vanishes the loss of weight is shown with a subsequent gain in weight. The enormous output of plasma protein is shown in Table 3 and it was found impossible to keep the usual low plasma protein level during the liver period. When the basal diet is again resumed the usual preponderance of globulin promptly appears.

DISCUSSION

The ratio of albumin to globulin in the total plasma protein concentration is of considerable interest and invites speculation. Few authors can resist this urge and too much space has been wasted on this elusive ratio. The normal ratio is about 2 parts albumin to 1 part globulin so it was logical to assume that these substances might be produced in about this same ratio. Experiments by many workers soon showed that as these substances were depleted the ratio might even be reversed so that the total plasma protein would show 1 part albumin and 2 parts globulin.

Because some workers recorded sudden shifts in this ratio due to relatively simple procedures, it was assumed by certain writers that albumin could be changed readily to globulin or *vice versa*. This did not seem to be reasonable because of known chemical differences between albumin and globulin and it is now believed that these earlier observations were in error due to method inaccuracies.

However it is well recognized that there may be a slow change in the albumin globulin ratio due to plasma depletion or even to diet alone (Table 1—Dog 32-394). It would appear that as the plasma proteins are used up their replacement may result in a more prompt output of globulin as compared with albumin. Certain diets may favor globulin production (vegetable protein) and others (liver) may favor albumin production (see Table 3 and Chart A).

Why should liver feeding favor new plasma protein regeneration and especially serum albumin production? There may be several reasons for this observation (Table 3). It was shown by Kerr, Hurwitz, and Whipple (10) that there was a *reserve storage* of material which could be thrown into the circulation after a short severe plasmapheresis and that a previous liver injury impaired this reaction. In Table 3 it is noted that the initial *reserve storage*, which is exhausted by continued plasma depletion, is made up of albumin and globulin in about equal amounts. It is possible that this *reserve store* may be in large part in the liver and that albumin or its parent substances are well represented.

Furthermore it is generally accepted that fibrinogen, the plasma globulin, is formed in the liver (9) and by no other body tissues. It is not too much to suspect that the liver is also largely concerned with maintenance of the total plasma protein concentration (22). Also the liver protein in the food is known to favor the production of another important protein—hemoglobin. All this argument then is in harmony with the recorded observations (Table 3) that liver feeding exerts a potent influence on the regeneration of plasma proteins and in particular on the production of the albumin fraction.

When a dog with depleted plasma protein level is placed on a favorable diet (liver or casein) there is a 3–4 day interval before the rapid regeneration and production of plasma protein is evident. After the favorable diet period terminates and the dog is put back on the basal ration the active production of plasma protein continues briskly for 5–7 days and tapers off slowly to the basal diet level. This we term the "carry over" from the potent diet.

Exactly the same reaction is noted in the study of hemoglobin regeneration in dogs on various diets (21). Evidently it takes time for the body mechanism to elaborate these proteins from the various food factors and certain materials are stored in reserve during such a favor-

able diet period, which stores are used up or exhausted in the subsequent basal diet period.

SUMMARY

When blood plasma proteins are depleted by bleeding and return of the washed red cells (plasmapheresis) the regeneration of new plasma proteins can be controlled at will by diet. The amount and character of protein intake is all important.

Liver protein and *casein* are efficient proteins to promote rapid regeneration of plasma proteins but some vegetable proteins are also efficient.

The blood plasma proteins are reduced by plasmapheresis close to the edema level (3.5–4.0 per cent) and kept at this level by suitable exchanges almost daily. The amount of plasma protein removed is credited to the given diet period.

A *basal ration* is used which is poor in vegetable protein (potato) and contains no animal protein. The dog on this ration can be kept in nitrogen balance but can produce only about 2 gm. plasma protein per kilo body weight per week. With liver or casein feeding this production can be increased three- or fourfold.

A *reserve* of protein building material can be demonstrated in the normal dog when its plasma proteins are depleted. In the first 3 weeks of depletion this reserve in excess of the final basal output may amount to 30–120 gm. protein. This may be stored at least in part in the liver. As much as 50 per cent of this reserve may be albumin or albumin producing material.

A reversal of the *albumin-globulin ratio* may be observed on the basal diet alone. The reversal will always follow plasmapheresis with the dog on the basal diet and the total plasma protein output will consist approximately of 2 parts globulin and 1 part albumin. Liver diet will raise the production and output of albumin and bring the ratio back toward normal. Albumin production may actually exceed the globulin output during liver diet periods. The change is less conspicuous with casein but in the same direction.

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BLOOD PLASMA PROTEIN GIVEN BY VEIN UTILIZED IN BODY METABOLISM

II. A Dynamic Equilibrium between Plasma and Tissue Proteins

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The statement that blood plasma proteins can be utilized freely in the body economy has an heretical flavor yet it seems difficult if not impossible to explain the experimental data given below on any other basis. Dogs receiving only sugar by mouth can be maintained practically in nitrogen equilibrium by suitable amounts of blood plasma given intravenously. We realize that these statements will be challenged but hope that the experimental data will be adequate to convince even the skeptic.

Body metabolism can undergo extraordinary adjustments under the stress of emergency and we see the body produce large amounts of new hemoglobin and red cells during anemia periods with the diet intake limited to sugar and inorganic salts of iron. More than 100 gm. new hemoglobin can be produced by the dog in a 2 week period and we must assume that the new hemoglobin comes from tissue protein breakdown and conservation. The details of this experiment have been reported (1) very recently. Furthermore, in anemia, during fasting periods alone, the dog is able to conserve products coming from tissue breakdown and produce a considerable amount of new hemoglobin (30–40 gm. per 2 week period). In this emergency the tissue or blood plasma protein must contribute to the formation of the much needed hemoglobin.

When the plasma proteins are depleted (plasmapheresis) in short experiments (4), a rapid appearance of new plasma protein (reserve) and a slow return of plasma protein concentration to normal will be

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observed during fasting periods. Evidently this new plasma protein is produced in the body with zero protein intake and must come from reserve stores of plasma protein producing material or from breakdown and conservation of split products coming from tissue proteins. Both mechanisms may well be concerned.

Another important example of this "give and take" between various body proteins within the body was reported by Davis and Whipple (2). Dogs poisoned with chloroform may show a liver necrosis involving two-thirds of all the liver parenchyma yet they can regenerate the liver back to normal on an intake of sugar alone. This will all take place within 10–12 days and represents a very large production of highly specialized liver cell protein coming from other body protein and tissue cells or perhaps in part from plasma protein.

One need not be surprised therefore in reviewing the tabulated experiments below which indicate that body metabolism can utilize a surplus of plasma protein made available by intravenous injection with zero food protein by mouth. If this mechanism comes into play so promptly in an emergency we must admit as a possibility that it may take place to a limited degree during normal metabolism.

Fifty years ago it was believed (Voit and others) that the plasma proteins had much to do with the nutrition of tissue cells but with the discovery that the amino acids were absorbed from the intestine and carried throughout the body attention was focused on these "building stones." It was assumed purely on negative evidence that the plasma proteins were inert substances, having nothing to do with tissue nutrition. This may be the proper time to revise somewhat our conception of the plasma proteins, their usefulness and fate in the body.

One may inquire whether this introduced protein is broken down to amino acids before it is utilized in the body. This question cannot be answered as yet but some evidence is at hand to indicate that the breakdown does not carry to the end stage of the amino acids as the liver would probably deaminize some of this material and thereby increase the urea nitrogen in the urine. The urinary N does increase when the plasma protein is fed by mouth (Table 22). For the present we may imagine that the intravenously introduced plasma protein is broken down to "intermediates" before incorporation into the body tissues. The same thing is observed (6) when hemoglobin is injected

into the anemic dog.   The animal conserves either dog, sheep, or goose hemoglobin up to 90–100 per cent and builds it up into new dog hemoglobin and red cells.

Methods

The two dogs used were maturing litter-mate male hounds with a normal adult weight of about 16 kg.   Each experiment is divided into four periods: a fore-period of 2–5 days sugar feeding; then two 7 day periods of intravenous plasma injection; and finally a 5 day after-period of sugar feeding.   The weight loss, urinary nitrogen, urinary protein, plasma protein, plasma albumin, plasma globulin, plasma non-protein nitrogen, and plasma and blood volumes were determined during each period.

Handling of Animals.—Each of the dogs was first vaccinated against distemper. Two or more intravenous injections of donor's plasma were given to accustom the animal to the procedure.   The dog was weighed daily in the morning before anything was done.   All food was withheld for 3 days to allow nitrogen elimination to reach a fasting level.   The dog was then catheterized and placed in a clean metabolism cage.   2 hours before this and each subsequent catheterization 50 gm. of dextrose in 300 cc. of water was administered by stomach tube to favor a diuresis which would wash out residual nitrogenous waste products.   Each succeeding day of the experiment the dog received this dose of sugar and water by stomach tube, usually between 2 and 3 o'clock in the afternoon.   On alternate days 15 gm. of kaolin were added to the dextrose solution in an attempt to prevent diarrhea. When the experiment was over the dogs were placed on kennel diet and were not used again until they had regained their former weight, usually periods of 6–8 weeks being required.

Collection and Analysis of Urine Samples.—The urine was collected daily around 4 p.m., this being the time of the stated catheterizations.   The metabolism cage was kept in the laboratory under close observation throughout the day and urine voided was put aside in a separate bottle containing 5 cc. of toluol.   A wire screen was placed beneath the floor of the metabolism cage to catch any hair or thick excreta.   In this way contamination occurred only when the dog defecated or vomited and urinated before the cage contamination was detected and cleaned out. The few times that this did occur were at night.   Care was taken to wash down the sides of the cage with distilled water.   The daily urinary output was diluted to a known volume (usually 500 cc.) and analysed for nitrogen and protein.   Nitrogen determinations were run in duplicate by the macro-Kjeldahl method.   Results that did not check within 1 part in 60 were repeated; this reduces the probable error to less than 1 per cent.   A modification of the gravimetric method of Folin and Denis (3) was used to determine urinary protein excretion.   One-tenth of the diluted urine was boiled for 15 minutes with 5 cc. of 5 per cent acetic acid in a 100 cc. centrifuge tube, centrifugalized at 2,500 R.P.M. for 5 minutes or longer, the supernatant fluid poured off, the precipitate stirred up with 50 cc. of 0.5 per cent

acetic acid, again centrifuged and the supernatant fluid discarded; the precipitate stirred up again in 50 cc. of 50 per cent alcohol and centrifuged for the third time. The supernatant fluid was again discarded, and the centrifuge tube stoppered and set aside for the next day's analysis which was added directly to the precipitate in the tube. At the end of each period (5–7 days) the precipitate was carefully transferred to a Kjeldahl flask and its nitrogen determined by the macro-method. The amount of the precipitate was so small by the usual gravimetric method that it is the belief of the authors that this modification not only facilitates the determination but also increases its accuracy. The prescribed method of Folin and Denis was used in the first experiment, and it was only with the greatest care that one could get anything like satisfactory checks, for the usual daily precipitate was weighed in tenths of a milligram. At no time during this first experiment was the precipitate heavy enough to give a reading by the Esbach method.

Supplemental Injections and Feedings.—During the periods of plasma injection a donor was bled 500 cc. into a flask containing 5 cc. of saturated sodium citrate, done usually in the morning. The blood was centrifugalized for 35 minutes at 3,000 R.P.M. in 100 cc. centrifuge tubes, the plasma drawn off with suction, measured, warmed to 40–45°C., and given by stomach tube or injected into the jugular vein as indicated in each experiment, approximately 15 minutes being required for the plasma to run into the vein. Sometimes during the injection of the last 100 cc. the dog exhibited jerky movements of the extremities but these could be controlled by stopping the flow of plasma for a minute or so. A noticeable acceleration and decrease in the volume of the pulse usually preceded these jerky movements. These jerky movements, and sometimes retching, were most frequently encountered during the first two or three injections; thereafter the dog seemed to tolerate the procedure better. Occasionally, in spite of all precautions, the dog vomited during the latter part of the injection or soon after it was over. When the dogs were returned to their cages in a few minutes they appeared perfectly normal in all respects.

All donors were large, healthy dogs, weighing 25 kg. or more, and a sufficient number were used so that no donor was bled over three times during any 2 week period. The blood hematocrit of the donor was determined at each bleeding and, if found to be below normal, supplemental liver feeding or a rest period was instituted. Emphasis is placed on these points for, in calculating the daily nitrogen intake, we have assumed that all donors' plasma contains not less than 6 gm. of protein per 100 cc. At no time did the donors show the slightest ill effect of these repeated fairly large bleedings and they always ate all of their liberal allowance of kennel diet. It is for these reasons that we feel justified in assuming that the donors' plasma contained not less than 6 per cent protein, a figure about 0.5 per cent lower than the average of several actual determinations.

EXPERIMENTAL OBSERVATIONS

During the plasma depletion experiments in the preceding paper, a large surplus of normal dog plasma was available. Recent experi-

ments (5) in the laboratory had interested us in renal thresholds for hemoglobin which are very distinct and measurable values. It was thought that possibly a plasma protein renal threshold might be demonstrated if the concentration of plasma protein was pushed up to very high levels. To our surprise we found that although large amounts of plasma protein were given intravenously several days each week there was practically no escape by way of the urine which showed only traces of protein. The plasma protein level could not be pushed up more than 50 per cent—the plasma protein was being removed from the circulation and not by way of the urine. Obviously this called for a study of the nitrogen intake and output to understand what was happening.

Preliminary Experiment, Dog 32-130.—Between November 14, 1932 and January 20, 1933, this dog received 3,875 cc. of normal dog plasma containing approximately 227 gm. protein. Injections of 150–250 cc. citrated plasma were given intravenously several times a week. Kennel diet of hospital table scraps was eaten throughout the entire period. The dog was immature and as it grew gained weight from 10 kg. to 14.5 kg. A litter mate as control reached the same weight on January 20. The dog was normal throughout. Total plasma protein at start was 6.32 per cent. December 1, plasma protein = 7.37 per cent. December 31, plasma protein = 7.65 per cent. January 6, plasma protein = 7.04 per cent. The urine was followed closely for protein and at times showed a strong trace, again a faint trace, and often was negative for protein. Obviously there was no significant urinary escape of these plasma proteins nor were the values for blood plasma protein concentration much above normal.

Table 21 gives in summary three experiments on the same dog (32-131) with suitable rest periods of 6–8 weeks between to permit of complete return to normal weight. The table shows the experiments in the order in which they were done but perhaps we may best consider the last or control experiment first. The dog received only 50 gm. dextrose and water by stomach tube daily. There is a steady loss in weight and a uniform negative nitrogen balance of 1.8–2.1 gm. daily. There is the usual concentration of plasma volume from 850 cc. to 450 cc. observed in fasting dogs. There is little change in values for total proteins in the plasma but the albumin-globulin ratio does change. At the start the dog shows a somewhat unusual A/G ratio 1.0 which subsequently falls to A/G 0.76 with a rise in globulin values. These figures are given in the *clinical history* of Dog 32-131 below.

The figures for total circulating plasma protein obviously show a considerable loss (48 per cent) from 49 gm. to 25 gm.

When we contrast this control experiment (Dog 32-131) with the first experiment, Table 21, there are many significant differences. During the 2 week period of plasma injection the dog receives 179 gm.

TABLE 21

Plasma Protein Given by Vein Utilized in Body.—Sugar Control
Dog 32-131.

Experimental periods	Days	Injected plasma N, daily average	Urinary N, daily average	Negative N balance, daily average	Urinary N as protein N, daily average	Total blood plasma protein	Circulating plasma protein period end	Weight at period end
First		Plasma protein by vein—sugar by stomach tube						
		gm.	gm.	gm.	gm.	per cent	gm.	kg.
Fore-period.............	5	0	1.649	1.649	0.0	5.25	33.0	15.44
Plasma protein 81 gm.......	7	1.86	2.165	0.305	0.011	6.94	47.3	14.78
"　　" 98 "......	7	2.33	1.476	+0.854	0.038	7.01	44.7	13.92
After-period.............	5	0	1.770	1.770	0.011	6.06	42.2	13.40
Second		Plasma protein by vein—sugar and fat feeding						
Fore-period.............	5	0	2.499	2.499	0.003	5.94	42.2	17.38
Plasma protein 112 gm......	7	2.565	2.446	+0.119	0.052	7.62	64.2	17.28
"　　" 103 ".....	7	2.366	2.436	0.070	0.021	8.46	59.3	16.31
After-period.............	5	0	2.606	2.606	0.003	7.33	51.9	15.75
Third		Sugar alone by stomach tube						
Control..................	5	0	1.826	1.826	0	6.77	48.9	17.90
"　..................	7	0	2.105	2.105	0	6.17	40.7	16.67
"　..................	7	0	1.897	1.897	0	5.98	37.8	15.57
"　..................	5	0	—	—	0	5.62	25.3	15.17

plasma protein and not more than 1 per cent of this escapes in the urine. About 10 gm. remains in the circulation or 5 per cent. If we compare this with the *control fall* in plasma protein of about 20 gm. we may say that another 10 per cent should be added making approximately 15 per cent protein which is accounted for. Therefore about 85 per cent of the injected protein is used up in the body metabolism and we note a *positive urinary nitrogen balance* between protein N intake and urinary N output. If we include the fecal N loss (about 0.8 gm.

daily) and the loss due to bleeding (blood volume and plasma protein determination) the total nitrogen balance is slightly negative and during this 2 week period there is a loss of 1.5 kg. body weight—compare with loss of 2.3 kg. in sugar control period. There is no significant change in the albumin-globulin ratio but a moderate rise in plasma volume and circulating plasma protein.

The second experiment, Table 21, shows a similar reaction with 215 gm. plasma protein injected and a slight positive nitrogen balance between protein N injected and urinary nitrogen. The dog consumed 885 calories daily of fat and sugar during the 1st week and 590 calories daily in the 2nd week. During this 2-week period there was loss of weight of 1.0 kg., most of this in the 2nd week—compare with the control on sugar alone of 2.3 kg. body weight loss. The plasma volume remains unchanged as does the albumin-globulin ratio. There is a gain of about 10 gm. circulating plasma protein.

Clinical Summary

Dog 32-131—See Table 21, first experiment.

March 3. Weight 17.2 kg., young mongrel hound. Plasma protein = 5.6%; albumin = 3.61%; globulin = 1.99%; N.P.N. = 20 mg.; plasma volume = 762 cc.; blood volume = 1,552 cc.; red cell hematocrit = 50%.

March 5–7. All food withheld. Weight fell to 16.3 kg.

March 8. Daily 50 gm. glucose + 300 cc. water by stomach tube. Catheterization to start metabolism experiment.

March 12. Plasma protein = 5.25%; albumin = 3.54%; globulin = 1.71%. Catheterization. Plasma injection begun.

March 14. Plasma volume = 629 cc.; blood volume = 1,234 cc.; red cell hematocrit = 49%.

March 19. Plasma protein = 6.94%; albumin = 3.98%; globulin = 2.96%; N.P.N. = 18 mg. Catheterization.

March 20. Plasma volume = 685 cc.; blood volume = 1,161 cc.; red cell hematocrit = 41%.

March 26. Plasma protein = 7.01%; albumin = 4.28%; globulin = 2.73%; N.P.N. = 24 mg. Catheterization.

March 27. Plasma volume = 637 cc.; blood volume = 1,043 cc.; red cell hematocrit = 38%.

March 31. Plasma protein = 6.06%; albumin = 3.89%; globulin = 2.17%; N.P.N. = 16 mg.; plasma volume = 696 cc.; blood volume = 1,209 cc.; red cell hematocrit = 41%. Final catheterization. No fecal contamination of urine during entire period. Dog put on kennel diet.

Second experiment—Table 21.

May 26.   Weight 19.1 kg.   Plasma protein = 6.51%; albumin = 4.10%; globulin
= 2.41%; N.P.N. = 23 mg.; plasma volume = 730 cc.; blood volume = 1,551
cc.; hematocrit = 52%.

May 25–27.   All food withheld; weight fell to 18.8 kg.

May 28.   Daily 50 gm. glucose with 300 cc. water by stomach tube.   15 gm. kao-
lin on alternate days.   Catheterization to start experiment.   Plasma protein =
6.33%; albumin = 4.03%; globulin = 2.30%; N.P.N. = 25 mg.; plasma vol-
ume = 736 cc.; blood volume = 1,446 cc.; red cell hematocrit = 48%.

June 2.   Plasma protein = 5.94%; albumin = 3.18%; globulin = 2.76%; N.P.N.
= 17 mg.; plasma volume = 710 cc.; blood volume = 1,420 cc.; red cell hema-
tocrit = 50%.

June 2–7.   30 gm. mayonnaise + 20 cc. cod liver oil in addition to glucose.

June 9.   Plasma protein = 7.62%; albumin = 4.68%; globulin = 2.94%; N.P.N.
= 15 mg.; plasma volume = 842 cc.; blood volume = 1,414 cc.; hematocrit =
40%.   Catheterized.

June 10.   25 gm. lard.

June 11–16.   50 cc. cotton seed oil by stomach tube; retained every day except
June 15.

June 12.   Gross contamination of urine with feces.

June 16.   Plasma protein = 8.46%; albumin = 4.88%; globulin = 3.58%; N.P.N.
= 18 mg.; plasma volume = 701 cc.; blood volume = 1,287 cc.; red cell hema-
tocrit = 44%.   Catheterized.

June 21.   Plasma protein = 7.33%; N.P.N. = 19 mg.; plasma volume = 708 cc.;
blood volume = 1,235 cc.; red cell hematocrit = 40%.   Final catheterization.
Placed on kennel diet.

Third experiment—Table 21.

August 2.   Weight 19.5 kg.   Plasma protein = 6.40%; albumin = 3.04%; glob-
ulin = 3.36%; N.P.N. = 16 mg.; plasma volume = 869 cc.; blood volume =
1,675 cc.; hematocrit = 48%.   Total fasting.

August 3.   Total fasting.

August 4.   Plasma protein = 6.20%; albumin = 3.15%; globulin = 3.05%;
N.P.N. = 9 mg.; blood volume = 1,858 cc.; plasma volume = 1,010 cc.; hema-
tocrit = 46%.   Catheterized at 11:00 a.m. to start experiment.

August 5–25.   50 gm. of glucose by stomach tube with 300 cc. of water.   15 gm.
of kaolin added on alternate days.

August 9.   Plasma protein = 6.77%; albumin = 2.90%; globulin = 3.87%;
N.P.N. = 8 mg.; blood volume = 1,413 cc.; plasma volume = 722 cc.; hema-
tocrit = 49%.   Catheterized at 11:00 a.m.

August 16.   Plasma protein = 6.17%; albumin = 2.60%: globulin = 3.57%;
N.P.N. = 13 mg.; blood volume = 1,220 cc.; plasma volume = 660 cc.; red
cell hematocrit = 46%.   Catheterized at 11:00 a.m.

August 23.   Plasma protein = 5.98%; albumin = 2.44%; globulin = 3.54%;

N.P.N. = 8 mg.; blood volume = 1,078 cc.; plasma volume = 632 cc.; hematocrit = 41%. Catheterized at 11:00 a.m.

August 25. Weight 15.2 kg. Plasma protein = 5.62%; albumin = 2.52%; globulin = 3.10%; N.P.N. = 9 mg.; blood volume = 792 cc.; plasma volume = 452 cc.; hematocrit = 43%.

TABLE 22

Plasma Protein Given by Vein Utilized in Body.—Control Plasma by Mouth
Dog 32-130.

Experimental periods	Days	Injected plasma N, daily average	Urinary N, daily average	Negative N balance, daily average	Urinary N as protein N, daily average	Total blood plasma protein	Circulating plasma protein period end	Weight at period end
First		Plasma protein by vein—sugar by stomach tube						
		gm.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*kg.*
Fore-period..............	2	0	1.718	1.718	0.0	7.33	45.2	13.86
Plasma protein 88 gm......	7	2.000	1.988	+0.012	0.022	7.33	46.0	13.18
" " 104 "	7	2.389	1.564	+0.825	0.005	8.60	52.2	12.49
After-period..............	5	0	1.895	1.895	0.003	6.28	36.8	11.76
Second		Plasma protein by vein—sugar and fat by mouth						
Fore-period..............	5	0	2.684	2.684	0.001	5.77	33.5	13.91
Plasma protein 109 gm......	7	2.485	1.869	+0.616	0.001	7.52	47.3	13.95
" " 120 "	7	2.750	2.614	+0.136	0.010	9.74	68.8	13.16
After-period..............	5	0	2.492	2.492	0.004	7.79	59.7	12.54
Third		Plasma protein and sugar by mouth						
Fore-period..............	5	0	2.212	2.212	0	5.55	35.3	14.32
Plasma protein 139 gm......	7	3.17*	3.130	+0.040	0	4.76	28.8	13.47
" " 125 "	7	2.85*	2.970	0.120	0	5.63	25.1	12.90
After-period..............	5	0	1.970	1.970	0	5.52	31.6	12.33

* Plasma protein given by mouth.

Table 22 shows three experiments on the same dog (32-130) with 6–8 week rest intervals for complete weight recovery. This dog is slightly smaller and was given more plasma protein by vein so the results are even a bit more striking. During the first experiment the dog received 192 gm. plasma protein by vein and 50 gm. glucose with water by stomach tube. The loss of protein in the urine was less than 1 per cent. There was a positive nitrogen balance between the nitro-

gen given by vein and that eliminated in the urine.   The fecal nitro-
gen was analysed and found to be 0.8 gm. per day and together with
the routine bleeding for analyses gives a total negative nitrogen balance.
The loss of body weight in these 2 weeks is 1.3 kg. and is to be com-
pared with the third experiment on the same dog with *plasma protein
by mouth* and a body weight loss of 1.4 kg.

The second experiment (Table 22) is even better as more protein is
given by vein (229 gm.) and more calories are taken by mouth with a
very small total weight loss of 0.7 kg.   During the 1st week a daily
caloric intake of 1,020 was attained (fat and sugar) but the dog re-
fused some of this mixture in the 2nd week and consumed only 680
calories daily.   There was no change in the albumin-globulin ratio
but a small increase in plasma volume and total circulating plasma
protein.

The third experiment (Table 22) is of considerable importance and
gives a different type of control.   The dog was fed plasma protein by
stomach tube in the amounts tabulated in addition to 50 gm. glucose
daily.   The plasma protein by mouth exceeds somewhat that given
by vein and the urinary nitrogen is definitely higher due to this protein
by mouth.   This suggests deaminization by the liver.   The loss of
body weight (1.4 kg.) exceeds considerably the loss in the second
plasma injection experiment on the same dog (0.7 kg.).   Evidently
the protein by vein is a little more completely utilized to form new
protein in the body than the same protein given by mouth.   The fecal
nitrogen was analysed and found to be 0.8 gm. per day, the same
amount recorded when the plasma was given by vein.   The plasma
volume fell from 700 cc. to 570 cc. or less and there was a change in
the albumin-globulin ratio due to the limited food protein intake.
The albumin-globulin ratio fell from 1.5 to 0.9 indicating probably a
more rapid production of globulin and a loss of total proteins with the
fall in the plasma volume.

Clinical Summary

Dog 32-130—See Table 22, first experiment.
Weight 15.3 kg., young mongrel hound.
January 21–23.   *All food withheld, weight fell to 14.1 kg.*
January 24.   Plasma protein = 6.63%; plasma volume = 700 cc.; blood volume
      = 1,385 cc.; hematocrit = 49%.   Catheterized to begin experiment.   50 gm.

of glucose with 300 cc. of water given by stomach tube every day until end of experiment.   15 gm. kaolin on alternate days.

January 26–February 9.   Plasma protein injections.

February 2.   Plasma protein = 7.33%; albumin = 4.70%; globulin = 2.63%; N.P.N. = 22 mg.; plasma volume = 628 cc.; blood volume = 1,105 cc.; red cell hematocrit = 42%.   Catheterized.

February 9.   Plasma protein = 8.60%; albumin = 5.48%; globulin = 3.12%; N.P.N. = 27 mg.; plasma volume = 607 cc.; blood volume = 964 cc.; red cell hematocrit = 37%.   Catheterized.

February 14.   Plasma protein = 6.28%; albumin = 4.16%; globulin = 2.02%; N.P.N. = 22 mg.; plasma volume = 586 cc.; blood volume = 944 cc.; red cell hematocrit = 38%.   Dog placed on kennel diet.

Second experiment—Table 22.

May 1.   Initial weight 15.8 kg.

May 1–3.   All food withheld. Weight fell to 15.0 kg.

May 3.   Plasma protein = 5.63%; albumin = 3.87%; globulin = 1.76%; N.P.N. = 29 mg.; red cell hematocrit = 48%.   Catheterization to start metabolism experiment.   50 gm. glucose in 300 cc. water given daily by stomach tube.   15 gm. kaolin added on alternate days.

May 9.   Plasma protein = 5.77%; albumin = 3.61%; globulin = 2.16%; N.P.N. = 18 mg.; plasma volume = 581 cc.; blood volume = 1,039 cc.; red cell hematocrit = 44%.

May 9–13.   50 gm. lard daily.

May 14.   Received no fat.

May 15.   Received 100 gm. mayonnaise; 40 cc. cod liver oil; 100 gm. karo corn syrup.

May 16.   Plasma protein = 7.52%; albumin = 4.51%; globulin = 3.01%; N.P.N. = 14 mg.; plasma volume = 629 cc.; blood volume = 1,220 cc.; red cell hemacrit = 45%.   Received 20 gm. mayonnaise, 10 cc. cod liver oil, 20 gm. karo.

May 17.   75 cc. of cotton seed oil.

May 18–20.   Received 35 cc. cotton seed oil daily with urinary contamination by vomitus on last day.

May 22.   40 cc. of cotton seed oil.

May 23.   Plasma protein = 9.74%; albumin = 5.92%; globulin = 3.82%; N.P.N. = 26 mg.; plasma volume = 706 cc.; blood volume = 1,146 cc.; red cell hematocrit = 36%.

May 28.   Plasma protein = 7.79%; albumin = 4.93%; globulin = 2.86%; N.P.N. = 21 mg.; plasma volume = 766 cc.; blood volume = 1,155 cc.; red cell hematocrit = 34%.   Dog placed on kennel diet.

Third experiment—Table 22.

June 20.   Weighs 16.6 kg. Plasma protein = 5.90%; albumin = 3.51%; globulin = 2.39%; N.P.N. = 20 mg.; blood volume = 1,482 cc.; plasma volume = 797 cc.

June 20–22. All food withheld. Weight fell to 15.5 kg.

June 23. Plasma protein = 5.51%; albumin = 3.36%; globulin = 2.15%; N.P.N. = 34 mg.; blood volume = 1,213 cc.; plasma volume = 684 cc.; hematocrit = 43%. 50 gm. dextrose, 3 gm. kaolin. Catheterized to begin metabolism experiment. 50 gm. glucose in 300 cc. water given daily by stomach tube with 15 gm. kaolin on alternate days.

June 28. Plasma protein = 5.55%; albumin = 3.17%; globulin = 2.38%; N.P.N. = 26 mg.; plasma volume = 636 cc.; blood volume = 1,064 cc.; hematocrit = 39%. Catheterized.

June 29–July 13. About 250 cc. normal blood plasma by stomach tube given daily.

July 5. Plasma protein = 4.76%; albumin = 2.58%; globulin = 2.22%; N.P.N. = 12 mg. Catheterized at 4:00 p.m. Blood volume = 1,060 cc.; plasma volume = 585 cc.; hematocrit = 45%.

July 12. Plasma protein = 5.63%; albumin = 3.37%; globulin = 2.37%; N.P.N. = 13 mg.; blood volume = 814 cc.; plasma volume = 445 cc.; hematocrit = 45%. Catheterized.

July 17. Plasma protein = 5.52%; albumin = 2.66%; globulin = 2.91%; N.P.N. = 14 mg.; blood volume = 821 cc.; plasma volume = 574 cc.; hematocrit = 45%. Catheterized.

DISCUSSION

There is great temptation to use these facts to speculate about the problems of edema and hypoproteinemia in human disease. Perhaps discretion has some merit and we may leave these observations to the clinical investigators to use as they see fit in the study of human material. Observations in liver disease should prove to be of unusual value.

It may be proper to inquire whether there is evidence that the plasma proteins are *rapidly* depleted by fasting and restored by heavy protein feeding. The first paper (Chart A) shows how promptly the low plasma protein level will be restored by liver feeding. Fasting usually does not modify the total protein concentration in the plasma and the albumin-globulin ratio may not be changed. But fasting does cause conspicuous shrinkage of the blood plasma volume and therefore the total amount of circulating protein may decrease to 70, to 60, or even to 50 per cent of normal and represents a considerable loss of plasma protein—relatively more than the loss of general tissue protein (weight loss). To bring evidence for a considerable degree of rapid fluctuation in plasma proteins directly referable to diet intake

or fasting is a possibility. It seems certain that over periods of several days the plasma protein in circulation can be decreased by fasting or increased by heavy protein feeding.

It will be noted that in all the plasma injection experiments (Tables 21 and 22 and clinical histories, Dogs 32-130 and 131) there is a fall in hematocrit of about 10 per cent and this represents a loss of about 100 gm. hemoglobin when we calculate for changes in blood volume. This is very close to the actual amount of red cells and hemoglobin removed during the course of the experiment for routine analysis of plasma protein, blood volume, and red cell hematocrit. Usually a dog would regenerate new hemoglobin and red cells promptly to make up this loss but this reaction does not follow. As a possible explanation we refer to evidence that blood transfusions may inhibit red cell regeneration. If simple plasma transfusion has this same effect it may have some bearing on a correct explanation of this phenomenon. At any rate it seems fair to say that none of the injected plasma protein is utilized to form new hemoglobin.

The albumin-globulin ratio is unchanged after a long series of normal plasma injections. This would indicate that the body uses both these proteins in about the same amounts as represented in the normal plasma—not using more albumin than globulin. When we observe that in forming new plasma protein especially on a low protein intake we may see a preponderance of globulin, this suggests that globulin is more easily formed than albumin rather than that albumin is used up more expeditiously.

This "dynamic equilibrium" may mean a tidal ebb and flow between tissue protein and plasma protein. One may embrace in this equilibrium the food proteins but there seems to be little question that food proteins contribute to tissue proteins and plasma proteins depending upon the immediate needs.

Without food protein both the plasma and tissue proteins are progressively depleted and it is of interest to note that the plasma proteins may be relatively more depleted than are the tissue proteins (refer to Table 21, fasting alone, Dog 32-131). With adequate or high protein intake both plasma and tissue protein are restored and again this change is most conspicuous in the plasma proteins (Chart A, Paper I). There is much evidence that plasma proteins are more labile substances than are tissue proteins.

SUMMARY

Large amounts of normal blood plasma can be given intravenously to normal dogs over several weeks without causing any significant escape by way of the urine. There appears to be no renal threshold for plasma protein even with high plasma protein concentration (9.7 per cent).

Dogs receiving sugar by mouth and plasma by vein can be kept practically in nitrogen equilibrium and it would seem that the injected protein must be utilized by the body. If this can happen in this emergency we may suspect that normally there is a certain amount of "give and take" between body protein and plasma protein.

Plasma protein *fed* by mouth under identical conditions shows the same general reaction as noted with plasma by vein but the urinary nitrogen is a little higher and suggests that the *injected* protein is utilized a little more completely to form new protein. The difference may be explained as due to deaminization in the case of protein by mouth.

During fasting periods the blood plasma proteins are used up and the total circulating protein may even decrease to one-half the normal level. The plasma protein concentration changes but little and the significant change is a shrinkage of plasma volume.

All these facts point to a *dynamic equilibrium* between tissue protein and plasma protein depending upon the physiological needs of the moment. In the absence of food protein the body can use material coming from one body protein to fabricate badly needed protein material of different character.

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PERIVASCULAR REACTIONS IN LUNG AND LIVER FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI INTO PREVIOUSLY SENSITIZED ANIMALS

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PLATES 19 AND 20

Certain reactions of rabbits to intradermal injection of living non-hemolytic streptococci have been studied in detail by Swift and his coworkers (1, 2), who demonstrated that such injections properly spaced induce a condition in all respects comparable to the sensitization of infection (tuberculin allergy).

Among the detectors of this condition is the lethal test, which consists of intravenous injections of living cultures in doses comparatively innocuous for normal animals, but which cause the death of about 50 per cent of sensitized rabbits in from 24 to 48 hours. Macroscopic postmortem findings in animals so dying include swelling and edema of lymph nodes and thymus, with gross hemorrhages into these organs, and also into the tricuspid ring, subperitoneal tissues, and elsewhere (1). Because lethal inocula regularly induce severe tissue damage, it has seemed of interest to determine what lesions, if any, might follow the administration of non-lethal doses.

EXPERIMENTAL

Method of Sensitization.—Sensitization of animals has been induced by repeated intradermal inoculation with appropriate doses of living 24 hour broth cultures of known sensitizing strains of non-hemolytic streptococci. In a few instances hemolytic streptococci, either living or as heat-killed vaccines, have been employed in addition. More frequently, however, indifferent Streptococcus Q 155 has been used, inasmuch as its sensitizing and shocking capacities have been thoroughly

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tested (3).   Sensitizing injections of non-hemolytic streptococci have been given over periods ranging from 3 to 10 weeks, with a total dosage of blood broth culture varying between 0.15 and 2.11 cc. (4).   Living hemolytic streptococci have been used in such minute quantities that a total of 0.00022 cc. of living culture has not been exceeded; but as much as 4.44 cc. of vaccine made with this organism has been administered to one group.   Vaccines of hemolytic streptococci have been prepared by heating at 56°C. for 1 hour the washed sediment from 24 hour infusion broth cultures, then resuspending the sediment in a volume of physiological salt solution equal to that of the original culture.   Following the preliminary intradermal injections, the intravenous inoculum has consisted of doses varying from 1 to 5 cc., never exceeding a total of 6 cc. altogether, and this total has been divided variously into one, two, or three doses, given a day or two apart. As the 5 cc. quantity forms a large fraction of the shocking dose (1), it has been infrequently employed; more often divided doses of 1 or 2 cc. have been given. Indifferent Streptococcus Q 155 has uniformly been employed for the intravenous treatment.

Controls.—Several sets of controls were studied.   One series of animals was sensitized by intracutaneous inoculation, then sacrificed without receiving any intravenous treatment.   A second set was treated intravenously from the beginning, with the same amounts of culture as were given to a group simultaneously undergoing intracutaneous sensitization.   Failure of hypersensitiveness to develop in this group confirms the observation of Schultz and Swift (5).   Other animals received only an intravenous dosage similar to that administered to sensitive animals at the termination of an experiment.   Finally, a small group was kept under laboratory conditions, without receiving any treatment whatever with bacteria.

The development and extent of sensitization was judged by means of the ophthalmic reaction (2).   In addition, graded doses of bacteria were administered intradermally, and the size and course of the resulting lesions were observed daily for 72 hours (1).   In the case of unsensitized controls, however, the intradermal inoculations were omitted, inasmuch as no increment of allergy, however slight, was considered desirable.

Preparation of Tissues.—Except for a small number which perished within 24 hours of the intravenous treatment, all animals were sacrificed on the day following the final injection unless otherwise noted.   Some were chloroformed; others killed by a sharp suboccipital blow.   Autopsies were performed at once, and small pieces of various organs—thymus, heart, lung, liver, spleen, adrenal, kidney, lymph node, and bone marrow—were fixed in Zenker-acetic acid mixture.   Paraffin sections were prepared and stained with methylene blue and eosin, and with Weigert-Van Gieson for elastica and connective tissue.   Sections were studied by

one of us (A. R. C.) who had no knowledge of the treatment previously administered to any animal.

The Characteristic Lesions.—The most characteristic microscopic changes in positively reacting animals were found in the lung, liver, spleen, lymph nodes, and bone marrow. Scattered throughout the sections of the lungs were numerous, more or less uniform perivenous lesions consisting of definite collars six to ten cells in thickness (Figs. 1 and 4). The vessel wall at the site of the lesions was moderately edematous, and in some instances showed separation of the elastic fibers; in nearly all there were proliferation and swelling of the endothelial lining. The predominant cell in the perivascular lesion was large and irregularly shaped, about two and a half times the diameter of a mature lymphocyte (see Fig. 5). The cytoplasm was either homogeneous and deeply basophilic, or finely vacuolated and more faintly basophilic, with gradations between these two varieties. The rather large nucleus was pleomorphic—round, indented, horseshoe-shaped, club-shaped, or elongated. The nucleus was vacuolated, and contained dense chromatin packed at or near the center, apparently forming one to three nucleoli; masses of chromatin were also condensed at the periphery. Occasionally, delicate threads could be seen bridging the vacuolated gap between the central and peripheral masses of chromatin. Engulfed in the cytoplasm of the more faintly basophilic cells, which were considered as more mature forms of the cell under consideration, pyknotic nuclei of polymorphonuclear leukocytes were occasionally observed.

In addition to these basophilic cells, the perivascular lesions contained mature lymphocytes, a few eosinophiles, and other granulocytes. It is interesting to note that there was no evidence of local tissue destruction in the lesions and neither fibrin deposition nor attempt at reparative processes.

In the liver there were similar perivascular aggregates of large basophilic cells, lymphocytes, and granulocytes, particularly around the small branches of the hepatic artery at the periphery of the lobules (Figs. 2 and 3). The larger and more fully developed lesions also encircled the adjacent branch of the portal vein and the bile duct. In addition, the large basophilic cells were also found free in the liver sinusoids, where they could easily be differentiated from the

ordinary Kupffer cells normally occurring in this position. Among both controls and sensitized animals, were found a few cases of so called spontaneous cirrhosis of the liver, consisting of typical periportal fibrosis, lymphocytic infiltration, and regeneration of liver lobules. In view of the absence of fibrous tissue and other evidences of repair from the latter, and because of the distinct differences in cellular architecture, no difficulty was encountered in differentiating the spontaneously occurring lesion from that experimentally produced.

The large cell with basophilic cytoplasm predominating in the perivascular lesions is found normally in lymph nodes, spleen and other lymphoid tissue. In the hypersensitive animals marked hyperplasia and congestion were noted in lymphoid structures, and large basophilic cells were found in abundance in the sinuses of this hyperplastic lymphoid tissue and also in the pulp of the spleen. In the few specimens of bone marrow secured from such animals they were also present in increased numbers. In lymphoid tissue, where these cells were not gathered into definite aggregates, their cytologic features were more constant; the cytoplasm was more uniformly basophilic and the nucleus tended to be more constantly round.

The Experimental Animals.—For convenience, the experimental animals, exclusive of controls, were divided into four groups.

Group I consisted of six animals, each of which received on one day only intradermal inoculations of sedimented growth from 5 cc. of 24 hour blood broth culture of *Streptococcus viridans* V 92. 2 weeks later, when ophthalmic tests were performed, only two of the six showed marked sensitization. After the lapse of a further week, intravenous treatment with the homologous organism was begun; each animal received twice daily 1 cc. of culture per 2.5 kilos body weight. Three subgroups of two animals each were formed; one received 1 day of intravenous treatment, one 2 days, and one 3 days. One of the animals having ophthalmic hypersensitivity died following the 1st day's treatment, and the other after 2 days'. The survivors were chloroformed. Unfortunately the material available from these animals was limited, as these experiments were made before this study was planned; no lung tissue is at hand, and hepatic tissue from one only. Cardiac, renal, and adrenal tissues were uniformly free from the lesion described above; in three of the six, however, the characteristic cells were present in increased number in thymus, spleen, and bone marrow. Two of these three animals showed no ophthalmic hypersensitivity, yet the liver from one of these two revealed the perivascular aggregates in moderate degree. Tissues from one of the sensitive

rabbits showed no striking alteration. Of the three reacting animals, two had been treated intravenously for 3 days, one for 2 days. In this group, therefore, the tissue reactions were apparently more closely dependent upon the prolongation of the intravenous treatment than upon the preliminary sensitization. It appears, however, from a consideration of Groups III and IV that the omission of lung and liver from the tissues is probably responsible for this apparent relationship.

Group II consisted of six animals treated intradermally over a period of 8 weeks with small doses of indifferent Streptococcus Q 155. The final injection was made intra-articularly (as part of another study). The total dosage of culture was 1.11 cc., distributed among eighteen inoculations. Ophthalmic sensitivity developed uniformly. 2 weeks after the intra-articular inoculation, each animal was given intravenously 1 cc. of 24 hour culture of Streptococcus Q 155. 2 days later three of the animals received a further intravenous injection of 5 cc. of similar culture. One animal from each subgroup died within 24 hours; two others were chloroformed after 24 hours, and two after 48 hours. Perivascular aggregates were uniformly present in the liver and lungs of all the animals except those dying spontaneously; and characteristic young cells were found in increased number in spleen and lymph nodes. Of the two rabbits which succumbed spontaneously, one showed marked aggregates about the vessels of the lung alone, without noteworthy increase of young cells in spleen or node; the other was quite free from perivascular reaction, and presented no unusual cellular picture in the lymphatic apparatus.

Serving in a measure as weakly sensitized controls upon this group, particularly with reference to the possible effect of intra-articular inoculation, were two rabbits, each of which was given on one day four intradermal injections of Streptococcus Q 155 aggregating 2.11 cc., and an intra-articular injection of 10^{-4} cc. in one case and 10^{-5} cc. in the other. Ophthalmic sensitivity failed to develop in one, and was very slight in the other. Following a 4 weeks' interval after the initial treatment, each animal received two doses of 2 cc. each of culture 24 hours apart. One animal was sacrificed after a further 24 hours, one after 48. No perivascular reactions were found, although in each case there was a slight increase in the number of basophilic cells in the spleen.

Group III consisted of five rabbits treated with hemolytic streptococci, Strain S 43 matt, avirulent for mice. Two animals were given intradermal inoculation of a total of 4.44 cc. of heat-killed vaccine over a period of 4 weeks. 2 weeks later a living culture of a hemolytic streptococcus strain, Q 33, was administered in dosage of 10^{-5} and 10^{-6} cc. intradermally; after 3 days living culture of Strain S 43 was similarly given. For eye tests, living hemolytic streptococci were inoculated with homologous immune rabbit serum. Such serum-treated organisms were found innocuous for normal rabbits' corneae, and were more satisfactory than vaccine for elicitation of the ophthalmic reaction. One animal showed marked sensitivity when such organisms were inoculated upon the scarified cornea; the other gave no reaction. 8 days following the last treatment with hemolytic

streptococci, each animal was tested with and found sensitive to indifferent Streptococcus Q 155; eye tests with this organism gave results comparable with those elicited with serum-treated hemolytic streptococci. 2 weeks after testing with Streptococcus Q 155, each animal received 1 cc. of culture of this organism intravenously, and after 48 hours was chloroformed. Marked perivascular reactions were present in liver and lung, and the spleens were found to be rich in basophilic cells. Two other rabbits of Group III received, from the beginning, living hemolytic streptococci (S 43) intradermally in doses of 10^{-5} and 10^{-6} cc.; a total of 0.000028 cc. of culture was administered over a period of 6 weeks. At the end of this period tests were carried out as above. Ophthalmic sensitivity to both Strains S 43 and Q 155, was intense; and cutaneous hypersensitivity to the latter was present. 2 weeks after testing with Streptococcus Q 155, 1 cc. of culture of this organism was administered intravenously, followed in 3 days by 5 cc. of similar culture. Both animals succumbed within 48 hours. No perivascular reactions were found in liver or lung, nor were the basophilic cells in the spleen increased in number. The final animal of this group received living hemolytic streptococci, one dose each of 10^{-5} and 10^{-6} cc. of culture intradermally, on two occasions 3 days apart. Strain Q 33 was employed for the first treatment, and S 43 for the second. 10 days after the second inoculation skin sensitivity to Strain Q 155 was already present. Eye tests were not done. 2 weeks later 1 cc. of culture of Strain Q 155 was given intravenously, followed in 3 days by 5 cc. 5 days after the second dose the animal was chloroformed. Perivascular reactions were present in both lung and liver, and there was a moderate increase in the number of basophilic cells in the spleen.

Group IV contained four rabbits sensitized by the intradermal route with green Streptococcus V 110 A and indifferent Streptococcus Q 155. During a period of 2 months each animal received a total of 0.22 cc. of culture of the former, and from 0.56 to 0.9 cc. of the latter. All developed ophthalmic reactivity to Strain Q 155. At the end of the period of sensitization, each animal received intravenously 1 cc. of culture of Strain Q 155, followed 24 hours later by a second dose of the same size. 24 hours thereafter the rabbits were killed by suboccipital blows. In each case perivascular reactions were found in liver and lung, with distinctly increased numbers of basophilic cells in spleen and lymph nodes.

The Control Animals.—Control Group A contained fifteen rabbits which received a total of 0.15 cc. of Strain Q 155 intradermally over a period of 6 weeks. No intravenous treatment was administered at any time. Seven developed satisfactory ophthalmic sensitivity. At the end of the period of sensitization the animals were killed by suboccipital blows. No perivascular reactions were found in the liver. In four animals slight perivascular aggregates were noted in the lung; these aggregates, however, were only two to three cells thick, contained fewer of the characteristic cells, and were found about only a few of the vessels in the section. In three, slight to moderate increases in the number of basophilic cells were noted in spleen, and in lymph node in four. Three of these four had shown satisfactory ophthalmic tests.

Control Group B consisted of seven rabbits treated intravenously with Strain Q 155, at the same time and in the same dosage as administered to the animals in Group A. No eye sensitivity developed. They were sacrificed at the same time and in the same manner as the animals of Group A. In one case slight perivascular aggregates were found in the lung; none was present in the liver. In two instances there were slight or moderate increases in the immature cells of spleen and lymph node.

Control Group C was composed of eight animals kept under laboratory conditions with Groups A and B, but uninoculated. They were similarly sacrificed, and at the same time. There was no ophthalmic sensitivity. In one animal there were slight perivascular pulmonary changes; in a second the spleen was rich in basophilic cells, while in a third these were present in considerable numbers in a lymph node.

Control Group D contained four rabbits sensitized over a period of 11 weeks with total doses of Strains Q 155 varying from 0.22 to 0.9 cc. All developed satisfactory ophthalmic reactions. No intravenous treatment was administered. None of these animals presented pulmonary or hepatic reactions, nor did spleen or lymph node contain increased numbers of immature cells.

In control Group E were three rabbits, each of which received intravenously 1 cc. of Culture Q 155 on each of 2 successive days. There was no preliminary sensitization. They were killed by suboccipital blows 24 hours following the second inoculation. One animal revealed slight pulmonary and hepatic perivascular reactions, together with a moderate increase in immature cells in the spleen.

DISCUSSION

Perivascular aggregates of basophilic cells, occasionally containing granulocytes, have been described by numerous authors in immunized or infected animals.

In previously vaccinated mice Tsuda (6) noted an acceleration of marked vascular endothelial swelling and perivascular accumulations of cells in foci induced by intracutaneous injection with streptococci or pneumococci. Domagk (7) described phagocytosis of staphylococci by swollen vascular endothelium of mice inoculated intravenously with these microorganisms; and this was followed later by focal accumulations of cells in several organs. Comparable perivascular alterations were noted by Louros and Scheyer (8) in mice that received streptococci intraperitoneally; and both they and Domagk observed that the number and intensity of reactions increased with repeated inoculations. Jacob (9), on the other hand, while recognizing the reactions in mice treated intravenously with staphylococci or green streptococci, failed to note this parallelism.

The evolution of vascular responses in guinea pigs was followed in more detail by Oeller (10) who described endothelial phagocytosis of avian erythrocytes 30 minutes after they were injected intravenously, and perivascular accumulations

of mononuclear cells after 60 minutes. He attributed the response to a toxic action of the foreign hemoglobin; but this opinion was controverted by Gerlach and his coworkers (11–13), who observed similar lesions in the lungs of supposedly normal animals. Epstein (14) noted particularly collections of "basophilic round cells" not phagocytic for carmine in the hepatic capillaries and periportal tissue of rabbits treated intravenously with sheep erythrocytes, swine serum, or lipoid serum mixtures. Pentimalli (15) observed similar accumulations of cells in various tissues of rabbits after repeated injections of foreign protein; most numerous in the liver, fairly frequent in spleen and lymph nodes, and least marked in the kidney and lung. These observations were confirmed by Vaubel (16) who also described fibrinoid swelling of the ground substance as often preceding the accumulations of mononuclear cells. He observed that the intensity of response was conditioned by the amount of foreign protein previously injected, or in other words, by the degree of hypersensitivity. Klinge (17) described a similar evolution of the focal lesions in rheumatic fever, and for this reason ascribed a common pathogenesis to the two conditions.

Siegmund (18) traced systematically the reactions in blood vessels following the introduction of a variety of substances, and found that certain dyes, colloidal metals, foreign proteins, or bacteria stimulated the active mesenchyme and induced focal myelopoiesis or lymphopoiesis in different degrees depending upon the nature of the stimulant. Following the intravenous injection of certain bacteria (19) there was marked endothelial activation, which was accelerated in highly immune animals and took the form of interstitial collections of lymphoid and plasma cells. Subsequently (20) he described the formation of "intimal nodes" in the blood vessels of rabbits and guinea pigs that received multiple intravenous injections of bacteria. Such nodes might heal completely or lead to atheroma-like lesions. Similar pictures were seen in the blood vessels of patients dying of typhoid fever or staphylococcal sepsis. In the veins and endocardium of subjects dying 6 to 8 weeks after the onset of scarlet fever there were subendothelial nodes, and in other structures areas of cellular proliferation closely resembling those of rheumatic fever (21). Scheyer (22) earlier had correlated the amount of proliferation of reticulo-endothelial elements in various sites with the clinical course in puerperal sepsis, and found that the presence of focal reaction indicated a certain degree of resistance to the infection. Intense mononuclear cellular proliferation was observed by Ehrich (23) and by Nye and Parker (24) in animals following prolonged intravenous injection of bacteria; and the latter described similar lesions after the injection of certain colloids.

It is obvious that lesions resembling those described by us have been elicited with a variety of antigenic reagents, introduced through different routes, and in several species of animal. Despite the inclusive use of the term "immune" in the German literature and also the description of "hyperergic inflammation" by numerous authors, there

has been little distinction drawn between conditions of immunity (*i.e.* lessened reaction with increased resistance to toxic agents) and sensitization (*i.e.* increased reaction to toxic agents either with or without increased resistance to those agents). Even when these factors are considered the probable differences in cytological reactions between "anaphylactic sensitivity" and the hypersensitivity of infection have often been ignored, particularly by most histopathologists. Dienes and Mallory (25), however, have described the differences between these two conditions, and their criteria will doubtless be more carefully applied in the future.

Böhmig and Swift (26) studied the cutaneous response to focal injection in "immune" and "hypersensitive" rabbits, and found in the former relatively less tissue destruction and granulocytic infiltration than in the latter. In all lesions there was a marked perivascular "monohistiocytic reaction." Böhmig (27) subsequently demonstrated a stage of "hypersensitive type of response" to focal inoculation during the early period of intravenous immunization, later an "immune type of response," and finally as the immunity passed off a return to the first phase. His further demonstration that an animal may show the "immune type of response" to the bacteria with which it has been immunized, but a "hypersensitive type" to heterologous bacteria suggests that it may be hypersensitive to one immunochemical component of a microorganism and immune to another, and that the final focal evidence of infection of a previously treated animal may be the algebraic sum of the various modes of response.

In view of the fact that various degrees and types of reaction may be elicited in different animal species by the same antigenic substance, and also that different antigens induce different responses in the same species, it would seem probable that among the numerous investigators most of the histologic permutations and combinations of allergy and immunity have been described. In spite of differences, however, in most instances there have appeared very similar perivascular aggregates. The production of this reaction may, then, be regarded as not dependent upon the presence exclusively of any particular allergic condition of the animal, but rather as a function of chronicity of treatment with antigenic material and also of dosage. In most of the papers cited, the doses, when mentioned, have been fairly large.

No claim is made, therefore, that the preliminary sensitization is a *sine qua non* for the elicitation of these peculiar perivascular aggregates. It becomes obvious, however, from a consideration of the

experimental data, that there are *quantitative* differences between the sensitive and normal animal. In other words, the rabbit, after preliminary sensitization by the intradermal route, developed this lesion following subsequent intravenous treatment with a dose that seldom induced a comparable lesion in a non-sensitized control. Furthermore, under the condition of the experiments, rabbits inoculated intravenously from the beginning usually failed to display the lesion. Because at certain periods this route of inoculation induces tissue "immunity" (hypoergy) rather than hyperergy (2, 27), the experiments here presented show that the development of perivascular collars is conditioned by tissue reactivity, in addition to being a function of dosage of antigen.

The failure of the lesion to develop in all rabbits showing positive ophthalmic reactions, as well as its occasional appearance in untreated animals, illustrates the variability of host susceptibility in this species. Rabbits become sensitized unequally; and ophthalmic hypersensitivity is no guarantee that the usually lethal test dose will be fatal. Many rabbits contract spontaneous streptococcal infections from which they recover, doubtless with altered tissue reactivity to subsequent infection. It is not surprising, therefore, that sensitization should not be equally effective in the individuals of any given group. Likewise, the occasional appearance of a perivascular collar in previously untreated rabbits may indicate either abnormal spontaneous hypersensitivity or previous infection. These host variations, in either direction, are quite familiar to immunologists.

It is noteworthy that in the four animals that succumbed to the intravenous injection of 6 cc. of culture no cellular multiplication was found, except for a moderate reaction in the lung of one; but microscopic congestion and hemorrhage occurred. Probably in these rabbits the condition of shock, due to excessive dosage of antigen, was sufficient to inhibit any proliferative response. In other words, although sensitized tissues are easily stimulated by small inocula to a rather typical cellular reaction, larger amounts of the same material may lead to cell death.

Spontaneously occurring lesions must be differentiated from those experimentally induced. In the rabbit periportal aggregates of small round cells mixed with a few fibroblasts are not uncommon, at times

associated with true portal cirrhosis; but the lymphocytes in such lesions are obviously mature, and the large deeply basophilic cells are not found.   On the other hand, these last mentioned cells do occur normally in spleen and lymph nodes, so that the extent to which reactions have been induced in these organs depends upon quantitative alterations in their cellular content, together with variations in cellular distribution.   Normally occurring peribronchial and perivascular nodules of lymphoid tissue are readily identified, from the architecture of the aggregate and the type of cell.

We have preferred describing rather than naming the large basophilic cell which predominates in the perivascular lesion, for with the data at hand attempts at classification would be futile.   The pleomorphism of its nucleus, the character and distribution of the nuclear chromatin, the nuclear vacuolations, the fine reticulation in the cytoplasm of the more faintly basophilic cells, and, finally, the occasional evidences of phagocytosis, all indicate the reticulo-endothelial system as its probable source.   Further study with vital staining methods and observation of its behavior towards parenterally introduced India ink should furnish more conclusive evidence as to its identity. Whether it is produced directly by the presence of the bacteria or secondarily to the destruction of some other cells or tissue is also a problem for the future to unfold.

SUMMARY AND CONCLUSIONS

Intravenous inoculation of small doses of non-hemolytic streptococci into previously sensitized rabbits is usually followed by the appearance of perivascular cellular aggregates in lung and liver.

The characteristic cell in these aggregates is moderately large, with vesicular nucleus, prominent nucleoli, clumped chromatin, and basophilic cytoplasm.   In addition, the lesions contain small lymphocytes and granulocytes.

This lesion is easily differentiated by architecture and cell content from normally occurring lymphoid aggregates, and from spontaneous rabbit hepatic cirrhosis.

This mononuclear response does not occur when the intravenous dose is large enough to cause death of the animal within 24 hours.

In spleen and lymph nodes the characteristic basophilic cells, which normally occur in these organs, are present in increased numbers.

Following intravenous treatment alone, or sensitization without intravenous treatment, the lesions occur much less frequently, and when present are smaller and more sparsely found.

Inasmuch as in the present series of experiments this lesion was not found in normal animals, and infrequently in those treated by the intravenous route alone, it is suggested that the preliminary sensitization serves to enhance the animal's reactivity to the antigen. In this way a small dose of bacteria is capable of eliciting the cellular phenomenon, which in unsensitized animals appears only when larger doses of antigen are administered over longer periods of time. Too large a dose of antigen, however, results in shock and cell death rather than proliferation.

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EXPLANATION OF PLATES

All tissues used in illustrations were stained with eosin and methylene blue.

PLATE 19

FIG. 1. Lung. Low power photomicrograph showing three perivascular aggregates in lung. × 100.

FIG. 2. Liver. Showing aggregate in portal area. × 200.

FIG. 3. Liver. Higher magnification, showing marked cellular aggregation around a portal vein. Numerous granulocytes are present. × 480.

PLATE 20

FIG. 4. Lung. Higher magnification. Endothelial reaction accompanying marked perivenous aggregation. × 480.

FIG. 5. Liver. A small portion of Fig. 3, showing pleomorphism of the characteristic cells (indicated by arrows). × 1,900.

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PLATE 19

PLATE 20

SINGLE CELL INOCULATIONS WITH TREPONEMA PALLIDUM*

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The development of syphilis in the rabbit following intratesticular inoculation of test material affords positive evidence of the presence of the virus in the inoculum. Failure to thus infect has been interpreted as indicating the absence of *Treponema pallidum* in the inoculum. Inasmuch as this interpretation is widely used in the study of experimental syphilis, it seemed desirable to test the validity of the assumption upon which it is based. The assumption is that if the virus is present in any quantity whatsoever it will lead to infection in the inoculated animal and that when infection does not result from such an inoculation it is because of the complete absence of *Treponema pallidum* in the inoculum.

When saline suspensions are made of the tissues of acute lesions in rabbits infected with syphilis the *Treponema pallidum* is usually, although not always, demonstrable by dark-field examination. Such preparations will lead to infection when injected into rabbits (the tissue transfer method of inducing infection). If a tissue suspension in which there are great numbers of visible organisms is subjected to a series of increasing dilutions with salt solution, a point is reached where no organisms are visible on careful and painstaking search, and yet the material will prove infectious when injected into rabbits. This observation is in accord with those of Truffi (1), Brown and Pearce (2), and Levaditi, Schoen, and Sanchis-Bayarri (3). Observations of this character have been cited as favoring the existence of a granular or ultramicroscopic form of the virus (3–5).

It is admittedly possible that visible *Treponema pallidum* are present

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in inocula inducing infection though not seen in the very small fraction subjected to dark-field study. Although this possibility does exist there seems to be no way to definitely prove or disprove it. Assuming that visible forms are always present, does this fact warrant the abandonment of the hypothesis of an ultramicroscopic form of the virus? If infection is produced only by the visible, morphologically typical forms of *Treponema pallidum*, how many organisms must be present to induce the disease in a susceptible rabbit? It has evidently been assumed by many workers in the immunity and chemotherapy of experimental syphilis that the quantity of virus is not an important factor in the initiation of disease. The tissue transfer method for determining the presence or absence of infection in an animal is based upon the assumption that if *Treponema pallidum* is present in the tissue it will induce disease when a saline suspension of this tissue is injected into another susceptible animal—and this without regard for the number of organisms present. Conclusions based upon this assumption are abundant in the literature. It has even been suggested that the method might be useful in determining either persistence of infection, or cure, in individuals who have undergone antisyphilitic treatment (6). Although in human syphilis the procedure, as a test for either the presence or absence of infection, has been shown to be unreliable (7), it is still widely used in the experimental disease.

Does a negative tissue transfer experiment indicate the complete absence of typical *Treponema pallidum* in the material being tested? In an attempt to throw some light upon this question varying dilutions were made of a testicular saline suspension which contained *Treponema pallidum*. Rabbits were then inoculated with the several dilutions. It was found that not only did dilutions in which there were no visible organisms give positive results but also that dilutions in which it seemed probable that *Treponema pallidum* was present, gave negative results. However, it was obvious that no conclusive data could be obtained by this approach. We therefore proceeded to determine whether syphilitic infection could be induced in the rabbit by the inoculation of from one to several morphologically typical organisms. It was thought that if infection resulted, the experiment would afford convincing evidence of the reliability of the tissue transfer method as a means of determining the presence of infection. If infection did not result

from such an inoculation, the broad conclusions based on results of the use of the tissue transfer test to determine the presence of infection are not warranted and we must conclude that either the capacity for initiating infection does not reside in the visible organisms when present in minimal numbers, or that the rabbit's ability to resist infection is adequate for organisms when present in minimal numbers. Moreover, it seemed possible that these inoculations might throw some additional light on theories pertaining to the existence of an ultramicroscopic or granular form of the virus.

With these considerations in mind we have inoculated rabbits with material containing *Treponema pallidum* in numbers varying from one to six organisms.

EXPERIMENTAL

The equipment consisted of a Chambers micro manipulator (8) and a special dark-field condenser with moist chamber.[1] Detailed descriptions of the micro manipulator and its adaptation for isolation of single bacteria in the light field have been published by Kahn (10) and Gee and Hunt (11). The dark-field condenser and moist chamber have been described by Hauser (12).

The dark-field condenser is of the bicentric type with a focal length of 10.7 mm. and a working distance of 14 mm. The range of aperture of the illumination rays is from 0.85 mm. to 0.99 mm., which is necessary for use with oil immersion objectives. The diameter of the condenser is 49 mm. and its height is 42.5 mm. It is provided with lateral slots 15.5 mm. wide and 10 mm. high to allow ample room for the vertical and horizontal movements of the micro needles. There is a depression in the base of the condenser which is filled with water to provide moisture in the chamber. A microscope with an opening in the stage of at least 50 mm. in diameter is required.

The moist chamber has a base formed by an 80 mm. square of glass containing a circular aperture in the center. This aperture is partially surrounded by the two curved glass walls of the chamber. The two openings in the walls are opposite each other. The chamber is roofed with glass in such a manner as to leave an open space across which a cover-slip with hanging drop may be placed.

In order to maintain proper moisture in the chamber its walls are lined with moist blotting paper except at the point where the pipettes are to be introduced. The top of the chamber is covered with a mica apron containing an opening slightly smaller than the cover-glass to be used. The apron is sealed to the chamber by vaseline and the cover-slip is likewise sealed to the mica apron by vaseline.

A carbon arc lamp and water filter is used. By properly focussing the condenser so that the light is concentrated at different levels in the hanging drop, and by

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[1] The dark-field condenser and moist chamber is manufactured by E. Leitz Co.

properly adjusting the diaphragm in the oil immersion objective, an excellent dark-field is obtained and the drop and its contents may thus be inspected.

The pipettes used in the experiments are prepared in the manner described by Kahn (13).

Cover-slips not exceeding 0.17 mm. in thickness are used and a fine grease film is applied to them in order to prevent spreading of the hanging drops.

Two strains of *Treponema pallidum* were used. One of these, the Nichols strain, was furnished us by Chesney in 1927 and has regularly produced syphilis in rabbits during the past 6 years. The other strain which is designated S in Table I, was isolated in 1932 (14) from a case of gastric syphilis. The lesions produced by it in the rabbit are, in every way, typical of the experimental disease. Animals infected with these strains were sacrificed at intervals of from 17 to 36 days. Saline emulsions of early testicular lesions were prepared in the usual way, and control animals were inoculated with the undiluted emulsions. The emulsions were then subjected to the dilutions found appropriate for single cell isolations.

A small amount of the suspension is aspirated into a 1 cc. syringe through a 22 gauge needle. A drop of this material is then examined in the dark-field and enough warm salt solution is added to obtain the optimum preliminary concentration of organisms. This can only be determined by experience. By means of a micro pipette drops of the diluted suspension are placed upon the under surface of the cover-slip in the moist chamber. This is rapidly inspected in order to determine the actual number of organisms present in each drop. When a drop is found which contains the desired number of organisms their morphology and motility is noted. If this is found to be normal the entire drop is rapidly aspirated into a sterile micro pipette containing salt solution. The site of the drop on the cover-slip is then carefully examined to be sure that the organism or organisms have actually been aspirated into the pipette. The latter is immediately removed from the manipulator. Its tip is introduced into an 18 gauge needle which is attached to a 1 cc. syringe filled with salt solution. The tip of the pipette is then broken off at the bend of the shaft. The needle is then introduced directly into the testicle of a rabbit and the glass tip with its contents is washed into the testicle by the injection of the salt solution. The testicle is massaged to distribute the material.

Eighteen rabbits were thus inoculated, sixteen with single organisms, one with two, and one with six organisms (Table I). These animals were carefully observed for evidence of the development of syphilitic infection over periods varying from 6 to 13 weeks. Each animal was then sacrificed. The testicle which had been inoculated, together with the inguinal lymph nodes when they were readily found, were ground in a mortar containing salt solution. The resulting saline suspension was subjected to dark-field examination and injected into the testicles of either one or two normal rabbits. These rabbits were in turn observed for from 4 to 8½ weeks for manifestations of syphilitic infection. At the termination of this period each

TABLE I

Rabbit No.	Date of inoculation	No. of *Treponema pallidum* inoculated	Strain of *Treponema pallidum*	Time interval before 1st tissue transfer experiment	Dark-field examination of inoculum	Rabbit Series No. 2	Time interval before 2nd tissue transfer experiment	Dark-field examination of inoculum	Rabbit Series No. 3	Time interval before sacrifice of animal or reinoculation experiment	Dark-field examination of testicular suspension	Reinoculation with homologous strain of *Treponema pallidum*
	1933			wks.			wks.			wks.		
4-01	May 27	1	S	6	N.*	4-01 A	7	N.	4-01 C	9	N.	
						4-01 B			4-01 D	8†		N.
4-02	Mar. 29	1	S	7	N.	4-02 A	7	N.	4-02 C	12		P.
						4-02 B			4-02 D	12		P.
	1932											
4-05	Apr. 8	1	Nichols	6	N.	4-05 A	4	N.	4-05 C	8†	N.	
						4-05 B	7	N.	4-05 D	23		N.
4-07	Apr. 8	1	Nichols	6	N.	4-07 A	7	N.	4-07 C	8†	N.	
						4-07 B			4-07 D	1†	N.	
4-08	Apr. 10	1	S	7	N.	4-08 A	7	N.	4-08 C	18†		P.
									4-08 D	12†	N.	
4-10	Apr. 14	1	S	8	N.	4-10 A	7	N.	4-10 C	10	N.	
						4-10 B			4-10 D	12	N.	
4-12	Apr. 19	1	Nichols	7	N.	4-12 A	7	N.	4-12 C	4†	N.	
									4-12 D	8†	N.	
4-14	Apr. 19	1	Nichols	8	N.	4-14 A	7	N.	4-14 C	17		P.
						4-14 B			4-14 D	17		P.
4-15	Apr. 19	1	Nichols	8	N.	4-15 A	7	N.	4-15 C	16		P.
						4-15 B			4-15 D	16		P.
4-16	Apr. 19	1	Nichols	13	N.	4-16 A	8	N.	4-16 C	10		P.
									4-16 D	10		P.
4-17	Apr. 29	1	Nichols	12	N.	4-17 A	5	N.	4-17 C	14		P.
						4-17 B			4-17 D	7†	N.	
4-18	Apr. 29	1	Nichols	12	N.	4-18 A	5	N.	4-18 C	1	N.	
						4-18 B			4-18 D	14		P.
4-19	May 3	1	S	11	N.	4-19 A	5	N.	4-19 C	14		P.
						4-19 B			4-19 D			
4-22	May 7	1	Nichols	6½	N.	4-22 A	7	N.	4-22 C	16		P.
						4-22 B			4-22 D	16		P.
4-24	May 19	1	Nichols	6½	N.	4-24 A	4	N.	4-24 C	16		P.
						4-24 B			4-24 D	2	N.	
4-25	May 19	1	Nichols	10	N.	4-25 A	4	N.	4-25 C	14		P.
						4-25 B			4-25 D	1	N.	
4-04	Apr. 8	2	Nichols	8	N.	4-04 A	7½	N.	4-04 C	4	N.	
									4-04 D	18		P.
4-09	Apr. 14	6	S	8	N.	4-09 A	7	N.	4-09 C	9		P.
						4-09 B			4-09 D	9		P.

* N., negative. P., positive.

† Animals dead or in poor condition.

pair of the second series of rabbits was sacrificed, the injected testicles ground in salt solution, pooled, and injected into the testicles of one or more normal animals. Dark-field examinations were also made of these suspensions. This third series of rabbits was carefully observed for evidence of syphilis. At the end of varying periods of observation all had remained normal. Twenty-one animals of this group were then reinoculated with the homologous strain of *Treponema pallidum*.

Evidence of syphilis failed to appear in any of the rabbits and no organisms were demonstrated in any of the testicular suspensions by dark-field examination. In those animals of the third series which were tested, no convincing evidence of immunity to the homologous strain was found; only two of the twenty-one rabbits reinoculated failed to develop typical syphilis (see Table I). The animals of the control series, which were inoculated with the usual quantity of undiluted virus, invariably developed syphilitic infection.

DISCUSSION

The consistently negative results of the sixteen experiments indicate conclusively that an inoculation with one morphologically typical, motile *Treponema pallidum* does not induce syphilis in the rabbit. In two other experiments infection did not occur following the inoculation of either two or six organisms.

This failure to infect can hardly be due to the method employed. The possibility that the organisms were not viable when injected seems remote. They were actually delivered into the testicles within a few moments after they had been seen to be normal morphologically and actively motile in the hanging drop. It seems highly improbable that the short bit of fine glass capillary pipette in which they were injected into the testicles and which was probably distributed by the subsequent testicular massage could have injured the organism or otherwise acted as a factor to prevent infection.

A more reasonable explanation of the failure to infect is to be found in a consideration of other possibilities. The resistance of the rabbit to the syphilitic virus is admittedly weak. However, as has been pointed out, we have seen injections with inocula which we felt certain contained *Treponema pallidum*, although not demonstrable by dark-field examination, fail to induce infection. That these inocula did actually contain organisms is highly probable in the light of the work of Jahnel and his associates (15) who found that blood suspensions containing 300 *Spirochaeta hispanica* per c. mm. gave negative dark-field findings but would infect mice when injected in 0.2 cc. quantities. Moreover,

these observers point out that suspensions containing 80 organisms per c. mm., or less, fail to induce infection when injected in 0.2 cc. quantities.   These observations indicate that an irreducible number of *Spirochaeta hispanica* must be present before infection can develop and it is probable in the light of our work that this also holds for *Treponema pallidum*.

Our work offers no support to the theory of the existence of an ultra-microscopic or granular form of *Treponema pallidum*.   Such negative evidence as our experiments afford cannot be interpreted as indicating that the morphologically typical, motile *Treponema pallidum* is avirulent.   It seems necessary to emphasize this since the proponents of this theory have cited positive tissue transfer experiments, with inocula in which organisms were not demonstrable by dark-field examination, as evidence favoring their hypothesis.   The negative results obtained by us with typical organisms in a menstrum in which the hypothetical virulent form may be said to have been lost by dilution, might lead to similar erroneous interpretations.

The assumption by workers in experimental syphilis that a negative tissue transfer experiment indicates the absence of *Treponema pallidum* in the inoculum and the absence of syphilitic infection in the source animal is not warranted.   This is indicated by the experiments reported herein as well as by a previously reported study of the tissue transfer method in human syphilis (6).

SUMMARY

Sixteen rabbits were inoculated intratesticularly with single *Treponema pallidum*.   Two other animals were inoculated, one with two, and one with six organisms.   All of these animals remained normal.   Control animals inoculated with the usual quantity of the same but undiluted virus developed typical lesions of experimental syphilis.   The test animals were subjected to a procedure designed to demonstrate the presence of *Treponema pallidum* even in the absence of recognizable syphilitic lesions.   At appropriate intervals transfers were made of testicular material from these to a second series and, in many instances, from the second to a third series of rabbits.   All of the rabbits remained normal.   Moreover, immunity to the homologous strain was not present in those animals of the third series which were tested.

The relation of these observations to (*a*) the theory of the existence of an ultramicroscopic form of *Treponema pallidum*, and (*b*) the assumption upon which is based the tissue transfer method of determining the presence or absence of syphilitic infection, is discussed.

CONCLUSIONS

The injection of one or several *Treponema pallidum* into the testicles of rabbits does not induce syphilitic infection.

A negative tissue transfer experiment does not preclude the presence of *Treponema pallidum* in the inoculum nor does it indicate the absence of syphilis in the source animal.

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RIFT VALLEY FEVER IN MAN

REPORT OF A FATAL LABORATORY INFECTION COMPLICATED BY THROMBOPHLEBITIS

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Although Rift Valley fever is a natural disease of sheep with a mortality of 50–95 per cent, it occurs also in man as an acute febrile illness, usually of such a mild character that in over 200 cases known to have occurred in British East Africa no untoward sequelae were observed (1). The purpose of the present communication is to report a case of laboratory infection—the first known instance of Rift Valley fever in America—which terminated in death.

Rift Valley fever or enzootic hepatitis occurs among sheep in the Kenya Colony of British East Africa (1, 2). Its symptoms are not striking; usually the affected animals show little more than listlessness, disinclination to feed, and progressive weakness. In older animals bloody diarrhea and thick mucoid rhinitis may also be observed. So rapid is the course that many animals are found dead without ever having been observed to be sick, and in most instances death supervenes within 24 hours after the initial sign of illness.

At autopsy the chief pathological change found is an extensive necrosis of the liver. In advanced cases, the liver is affected to such an extent that stained sections of it are scarcely recognizable as hepatic tissue. The lobules consist merely of irregular shaped masses of broken-down, faintly staining cells intermingled with polymorphonuclear and mononuclear leucocytes; the Kupffer cells can no longer be distinguished. In less advanced cases, the lobules of the liver are still distinguishable, but are studded with areas of necrosis of different sizes. About the periphery of these foci of degeneration are found parenchymatous cells containing cytoplasmic hyaline bodies similar to those described originally by Councilman (3) and more recently by Klotz and Belt (4) as occurring in the livers of human beings dead of yellow fever. Acidophilic intranuclear inclusions are found in great number, which, although usually of less definite outline and not so strongly acidophilic, resemble those associated with herpes and Virus III. In addition to the lesions in the liver, the ileum and large intestine are often the seat of a hemorrhagic enteritis associated with congestion of the mesenteric and omental vessels.

Hemorrhages in other organs, especially in the cortex of lymphatic glands and beneath the capsules of the spleen and the kidneys, are also common.

The etiological agent of Rift Valley fever has been shown by Daubney and Hudson (1) and others to be a filterable virus which invades the animal so completely that it can be recovered from almost all organs of the body. In addition to sheep, natural outbreaks have occurred among cattle and, as has been stated, spontaneous cases among human beings are common during the epizootics. The disease has been produced experimentally in monkeys, goats, cats, rats, and in several species of mice. Recovery from the disease apparently confers a lasting immunity.

Rift Valley Fever in Man

During the original investigations (1) of Rift Valley fever in Africa all four of the Europeans engaged in the work developed an acute febrile illness characterized by general malaise, joint pains, and vague abdominal tenderness. Blood taken from the workers at the height of their fever produced typical Rift Valley fever when injected into lambs. Upon inquiry it was then learned that almost every native engaged in herding sheep during the epizootic had been sick for some days and had complained of fever accompanied by severe pains throughout the body. These illnesses were considered most probably to have been attacks of Rift Valley fever. A native volunteer was then inoculated with the virus. 3 days after inoculation he developed headache and pains in the back, and, a day later, fever. Blood taken from him at this time caused Rift Valley fever when injected into lambs. Following the investigations in Africa, the virus was sent to England for further study. Soon after initiation of the work there, three laboratory workers developed the disease (2). Up to the present time the attacks in the eight individuals mentioned represent the only well observed cases of Rift Valley fever recorded in the literature. The following description of the disease is compiled from the reports of these cases.

The course of Rift Valley fever in man is much like that of a mild attack of influenza. Following an incubation period of 5 or 6 days, the patient complains of general malaise, chilly sensations, and headache. After 6–12 hours of increasing symptoms the temperature, which has been normal, rises rapidly to 102–104°F. With the onset of fever, the patient may develop chills. Pains spread into the extremities and joints; a sensation of fulness over the region of the liver may be followed by definite tenderness or even abdominal pain. Nausea and vomiting

sometimes occur, and in one instance epistaxis has been recorded.   On examination the patient is found to be moderately prostrated.   The face is flushed; conjunctival injection and photophobia may be present; the tongue is coated; the breath is foul. The lungs are normal, but there is definite tenderness in the epigastrium.   The liver and spleen cannot be felt.   Although fever and symptoms may persist as long as 10 days or no longer than 1, improvement is usually evident about the 3rd day and proceeds rapidly to recovery.   In one case there was a second temperature reaction accompanied by a return of symptoms 3 days after recovery from the initial attack.   At the onset of the symptoms there may be a slight polymorphonuclear leucocytosis.   This gives way at about the time of appearance of fever to a leucopenia, the total white blood cell count reaching 3000–4000.   The fall in the number of leucocytes is almost entirely due to a decrease in the number of the polymorphonuclear elements.   The return of the leucocyte count to a normal level is slower than the clinical improvement.   Despite the period of probable liver damage in Rift Valley fever in man, the bile pigments of the blood serum and urine are usually not appreciably increased in amount.

Report of Case

A pathologist, R. S., age 30 years, had been working with Rift Valley fever virus for several weeks before the onset of his illness.   On the evening of December 22, 1932, he felt chilly while walking home and complained that his eyes and the calves of his legs ached.   He found his subglossal temperature at that time to be 99.6°F. During the night his rest was disturbed by general malaise and pains, especially around the knees and hips.   On awakening, the 2nd day of illness, his temperature was 101°F.   He attempted to continue his work but had several chills during the day and felt so miserable that he took to bed.   He complained at this time of a vague soreness over his abdomen, constant dull headache, and pain behind the eyes associated with slight photophobia.   There was no sore throat, rhinitis, nausea, or vomiting.   He was admitted to the Hospital of The Rockefeller Institute 24 hours after the onset of symptoms.

On admission, the temperature was 102.6°F., pulse 140, respirations 24.   The patient was definitely prostrated by his illness but rational and cooperative. There were found on physical examination a slight injection of the throat, and a vague tenderness over the abdomen.   The edge of the liver was not felt.   Other than this the examination was entirely negative.   The total leucocyte count at this time was 2800 per c. mm.   Polymorphonuclear elements constituted 57 per cent of this; small lymphocytes 9 per cent; intermediate lymphocytes 10 per cent; monocytes 24 per cent.[1]   The throat culture was negative for hemolytic streptococci and influenza bacilli, and blood cultures in infusion broth remained sterile. The urine showed a faint trace of albumin; no bile was present.   A tentative diagnosis of Rift Valley fever or influenza was made.

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[1] All differential counts were made on supravitally stained specimens.

In an attempt to confirm the tentative diagnosis of Rift Valley fever, 6 cc. of the patient's blood, drawn on the day of admission and kept fluid by the addition of 1 cc. of 1:1000 heparin solution, was injected intraperitoneally in 1 cc. amounts into six mice.   All of the mice died within 48 hours.   Their livers were free from ordinary bacteria, but showed in hematoxylin-eosin-stained sections the marked focal necrosis and acidophilic intranuclear inclusions typical of Rift Valley fever. Moreover, the disease was transmitted to other mice by means of serial passages. Blood taken from the patient on the day following admission gave similar results.
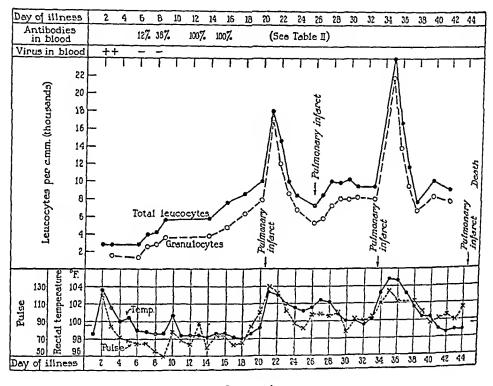
CHART 1

Consequently, the diagnosis of Rift Valley fever was confirmed.   In Table I and Chart 1 are shown the results of these and subsequent tests for the presence of virus in the patient.

Almost immediately after admission the patient began to improve.   The temperature, which reached a peak of 103.8°F. on the night of admission, fell promptly to normal within 24 hours (Chart 1).   The symptoms, however, abated somewhat less rapidly.   On the 5th day two papular areas several centimeters in diameter were observed on the right thigh and leg.   They resembled in a general way isolated measles papules.   After 3 days they had completely faded and since no others were observed at any time it is difficult to say whether the eruption bore

any relation to the disease. At the time of the appearance of the papules the patient developed a sore throat. The pharynx was irregularly injected and resembled the throats often seen in cases of influenza. On the following day (6th day of illness) a typical herpes simplex eruption appeared on the right side of the nose. Both this and the sore throat subsided in the next few days. During all this time the patient had been improving generally, complaining for the most part only of a constant feeling of fulness in the epigastrium which he believed was aggravated by a persistent constipation. There was a very slight and unexplained rise in temperature to a peak of 100.8°F. during the 10th day of illness.

By the 12th day of illness the patient had improved to such an extent that he was allowed to sit in a chair for a short time and on the following day was permitted to walk a short distance. Thereafter, as his strength improved, his activity was increased. On the evening of the 16th day, however, he complained of pain in

TABLE I

Tests to Determine Presence of Virus in Patient

Day of disease	Material tested	Mouse test	Result
2	Whole blood	6/6	Positive
3	" "	6/6	"
6	" "	0/6	Negative
8	" "	0/6	"
22	Sputum	0/2	"
45 (death)	Liver	0/3	"
45 (death)	Mesenteric lymph gland	0/3	"

All material was injected intraperitoneally into mice.

The denominator of the fractions signifies the total number of mice injected; the numerator denotes the number which died.

the dorsal part of the left leg. A diagnosis of phlebitis of the popliteal vein was made. The patient was returned to bed and the leg was immobilized in an elevated position. 4 days later (20th day of illness) the patient awakened with pain in the right chest which was more pronounced during deep inspiration or exhalation. Although the physical and X-ray findings in the chest were entirely negative at this time, it became apparent as conditions progressed that the patient was suffering from a small pulmonary infarct. Fever developed (103.4°F.); the leucocytes in the blood rose rapidly to 18,000 per c. mm. of which 94 per cent were granulocytes (Chart 1); dulness was apparent on percussion over the base of the right lung where the breath sounds, accompanied by many fine and coarse râles, were diminished. In the X-ray photograph made at this time there was a distinct shadow at the base of the right lung. On several occasions blood was expectorated in small amounts. In order to test for the presence of Rift Valley fever virus and

virulent pneumococci mice were injected intraperitoneally with the sputum but all remained alive and well (Table I).

During the following days the condition in the chest improved gradually until the 26th day when the patient developed a second pulmonary infarct in the right lung (Chart 1). Within a few days the patient began to improve again, but on the 34th day a third pulmonary infarct occurred, this time in the left lung. The temperature which had just returned to normal, rose rapidly to 104.4°F.; the leucocytes reached 24,200 with 90 per cent granular elements. By this time the patient had become very weak because of the repeated pulmonary insults. In spite of this fact, however, the temperature and leucocyte count again began a return to normal, and there was slow but progressive general improvement in the patient's condition. On the 38th day of illness definite signs of phlebitis in the right femoral vein developed. This caused no systemic reaction, however, and recovery seemed to be proceeding uneventfully. On the morning of the 45th day of illness, however, the patient suddenly collapsed and died within a few minutes. Death was apparently due to a large embolus in the pulmonary vessels.

At autopsy, only changes directly associated with the phlebitis and pulmonary infarcts were found. No abnormalities such as have been observed in animals dying of Rift Valley fever were seen. This is not surprising, however, since the patient died 45 days after the onset of his illness of which the acute stage occupied only the initial week. For this reason the pathological findings will be described but briefly. There were signs of a mild saphenous and femoral phlebitis. However, in the inferior vena cava where it receives the hepatic veins, there was a large thrombus branching out into the hepatic venous radicles and extending upward almost to the heart. The superior portion of this thrombus had apparently become detached, and had travelled through the chambers of the heart because it was found as an embolus in the pulmonary artery. In addition there were old pulmonary infarcts in both lungs with acute and chronic pleuritis. The liver appeared normal. Examination of the stained sections of the tissues revealed nothing unusual. Emulsions made from bits of liver and from mesenteric lymph glands were injected intraperitoneally into mice in order to test for the presence of Rift Valley fever virus. All the mice remained well (Table I).

Tests for the Presence of Antibodies in the Patient's Serum

At intervals during the course of the disease, blood was drawn from the patient in order to determine the rapidity with which antibodies to the virus are formed. To this end serum was collected on the 6th, 8th, 12th, and 15th days of illness and tested in the following manner. Decimal dilutions of infected mouse blood—ranging from 10^{-3} to 10^{-6} —were prepared. 0.5 cc. amounts of each dilution of virus were added to 1.25 cc. amounts of each specimen of serum to be tested. Without incubation, 0.7 cc. of each mixture were injected intraperitoneally into

each of two mice.   The mice were observed for 2 weeks to determine the mortality rate.   From the results shown in Table II it can be seen that antibodies were present in small amounts in the patient's blood as early as the 6th day and that by the 12th day had reached such a concentration that protection was afforded the mice against at least 1000 M.L.D. of the virus.   These findings are in agreement with those of Findlay (2) and Broom and Findlay (5).

TABLE II

Test to Determine Presence of Antibodies in Patient's Blood

Sera	Day of disease	Virus dilution				Total	Mice protected
		10^{-2}	10^{-4}	10^{-5}	10^{-6}		
							per cent
Negative control		2/2	2/2	2/2	2/2	8/8	0
Patient's serum	6	2/2	2/2	2/2	1/2	7/8	12
" "	8	2/2	2/2	1/2	0/2	5/8	38
" "	12	0/2	0/2	0/2	0/2	0/8	100
" "	15	0/2	0/2	0/2	0/2	0/8	100

The denominator of the fractions signifies the total number of mice injected; the numerator denotes the number which died.

DISCUSSION

For purposes of discussion, the course of disease in this case may be divided into two phases—the acute illness and the period of complications.   Concerning the first or acute stage, little need be said because the symptoms and signs differed in no essential detail from those previously described as characteristic of Rift Valley fever in man.   In fact, with such a typical syndrome, following definite exposure to the specific virus which in turn was isolated from the blood of the patient, there can be little doubt as to the true nature of the illness.   The diagnosis is further strengthened by the demonstration in the patient's blood of specific virus-neutralizing antibodies which during the early days of convalescence increased in amounts to reach a concentration capable of neutralizing at least 1000 M.L.D. of the active agent.

The phlebitis, however, which appeared during convalescence, came as an unexpected complication.   The condition is not mentioned in the papers which describe the clinical course of Rift Valley fever (1, 2)

and Daubney has reported (6) that enquiries concerning its occurrence in the Rift Valley have failed to elicit any affirmative information. However, it is not altogether surprising that phlebitis should occur during convalescence from Rift Valley fever, since it has been reported following almost all other known acute infectious diseases. Its relatively high incidence in pneumonia—0.72 per cent (7)—and in typhoid fever—2 per cent (8)—is well known. In the diseases thought to be due to filterable viruses it also occurs but less frequently. Thus, phlebitis has been reported following measles (9), mumps (10), varicella (11), smallpox (12), vaccinia (13), influenza (14), and psittacosis (15). What factors predispose a patient to this complication is not known, and no attempt will be made at this time to discuss the condition further than to point out its definite though infrequent occurrence in association with most acute infectious diseases. It seems probable that should the number of cases of Rift Valley fever increase, phlebitis will again be seen as a complication in a certain percentage of them.

SUMMARY

A case of Rift Valley fever following an accidental laboratory infection, and believed to be the first instance of the disease in the Western hemisphere, is reported. Although the course of illness was otherwise quite typical, it was complicated by thrombophlebitis—a condition not previously described in association with this disease in man. Death was caused by a pulmonary embolus.

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THE VITAMIN B_1 AND B_2 G CONTENT OF LIVER EXTRACT AND BREWERS' YEAST CONCENTRATE

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Recent developments in the study of specific therapy in pernicious anemia have led to the conception that two factors are responsible for the production of the anti-anemic substance which is normally stored in the liver, and which is responsible for the therapeutic benefits obtained from the administration of liver or liver extract. This view, as set forth by Castle (1) and now generally accepted, supposes (*a*) an intrinsic factor present in the gastric juice of normal persons but absent in the gastric juice of patients with pernicious anemia, and (*b*) an extrinsic factor present in certain articles of diet such as beef muscle. It has been shown that if normal human gastric juice is incubated and then fed to a patient with pernicious anemia no remission of the disease occurs. If beef muscle alone is fed to such patients no remission ensues. However, if normal human gastric juice and beef muscle are incubated together and then fed, a remission of the disease is brought about.

Little is known of the nature of the intrinsic factor, but Strauss and Castle (2) have tentatively identified the extrinsic factor as vitamin B_2 G. This conclusion is based on experiments in which human gastric juice and a brewers' yeast concentrate, vegex, were incubated together, and fed to patients with pernicious anemia. Remissions of the disease occurred under such treatment. Such remissions were obtained even when the brewers' yeast concentrate had been autoclaved at 15 pounds pressure for 5 hours prior to its incubation with normal human gastric juice. Since brewers' yeast concentrates, such as vegex, are supposed to contain vitamins B_1 and B_2 G (3, 4) and since the substance in vegex proved to be heat-stable, these workers concluded that the extrinsic factor was not vitamin B_1, which is heat-labile, but that it was probably vitamin B_2 G.

Since the experiments mentioned obviously did not involve the absolute identification of the extrinsic factor as vitamin B$_2$ G, it seemed important to approach the question from another angle; namely, to determine both qualitatively and quantitatively the vitamin B$_1$ and B$_2$ G content of this particular brewers' yeast concentrate, since it is known that brewers' yeast concentrates vary in their content of these vitamins (3, 4). As a corollary to this study, determinations were also made of the amounts of these two portions of the vitamin B complex in liver extract.

To ascertain the presence in foodstuffs of the vitamin B complex or the individual components making up that complex a standardized procedure is followed. Young rats of the usual laboratory strains weighing between 40 and 50 gm. are used as test animals. They are fed a basal ration which contains all the components required for normal growth except the vitamin in question. When growth no longer occurs on this diet the vitamin reserve of the animal body is considered to be exhausted and the substance to be tested for vitamin content is added to the ration. If growth then takes place at a normal rate for a sufficiently long period of time evidence of the presence of the vitamin in question is considered to be at hand. Unfortunately, different rates of growth have been considered normal by different investigators. Scheunert and Scheiblich (3) were satisfied with a weekly gain of 3.0 gm. when testing brewers' yeast concentrates for the presence of vitamin B complex. Later Quinn, Whalen, and Hartley (4) used the same rate of growth in tests of the same material for the components of the vitamin B complex, vitamins B$_1$ and B$_2$ G. Both groups of workers concluded that the content of vitamin B complex in brewers' yeast varied considerably. Furthermore, it was thought that the content of antineuritic vitamin B$_1$ was more variable than was the content of the thermostable vitamin B$_2$ G. In comprehensive and detailed experiments Chick and Roscoe (5) demonstrated that the rate of growth (3.0 gm. weekly) obtained in the experiments described above did not even approach the normal growth rate for the experimental animals employed. They proposed that a diet should be considered adequate only when an average gain of from 10.0 to 12.0 gm. weekly occurs. Levene (6) extended the observations of Chick and Roscoe by studying the normal rate of growth of rats on adequate diets. He found that the average normal monthly gain in white rats of 50.0 gm. weight was 52.0 gm. However, it was shown that the rate of growth varied somewhat at different seasons. During July and August the average monthly gain was only 29.0 gm., whereas in September and October it was 72.0 gm.

The presence of the vitamin B complex in fresh liver and liver extract has been known for some time (7, 8). Review of the published work indicates that inadequate information is at hand as to the content in these materials of the individual components of the vitamin B complex, vitamins B$_1$ and B$_2$ G. Guha (9–11)

was the first to test liver extract for its content of vitamin B_2 G.   A basal diet was used which contained vitamin B_1 but which was deficient in vitamin B_2 G.   A normal rate of growth was obtained (10.0 to 12.0 gm. weekly) when 40.0 to 60.0 mg. of liver extract, Lilly No. 343, an amount derived from 1 to 1.5 gm. of whole liver, was added to the diet.   If the material containing vitamin B_1 was omitted from the diet a normal rate of growth was not obtained, even if as much as 120.0 mg. of liver extract was administered daily.   From these studies it was concluded that liver extract was deficient in vitamin B_1 but contained a considerable amount of vitamin B_2 G.

Gilroy (12) ascertained the presence of the vitamin B complex in a variety of commercial liver extract preparations.   He used rats maintained on a diet deficient in the vitamin B complex.   Adequate growth was considered to be about 10.0 gm. gain per week for rats of 40.0 to 50.0 gm. in weight.   Normal growth was obtained when he added daily to the basal ration an amount of liver extract equivalent to 16.0 gm. of fresh liver.   If only half that amount of liver extract was employed, subnormal growth was obtained.   No attempt was made to determine which of the two components, vitamin B_1 or B_2 G, was deficient.   From the studies mentioned it has been assumed that liver extract is rich in vitamin B_2 G but deficient in vitamin B_1.   West (13) has prepared a substance from liver extract which produces typical remissions in patients with pernicious anemia. This material has been shown by rat tests to be lacking in vitamin B_2 G content.

Methods

The rats used in these experiments were all of the Wistar strain.   They were supplied through the Wistar Institute, or by Dr. H. M. Evans.   Immediately after weaning, all the animals were placed on a standard basal diet which was vitamin B-free.   It was of the following composition as recommended by Rose *et al.* (14):

Casein (extracted free from vitamin B)............................ 18
Salt mixture
   NaCl.. 3
   $CaCO_3$... 1
Butter fat... 8
Corn-starch... 48
Cod liver oil.. 2

In order to determine the presence of vitamin B_1 in the substances studied it was necessary to use a diet adequate in vitamin B_2 G.   This was supplied by adding to the basal ration twenty parts of bakers' yeast (Fleischmann) neutralized and autoclaved at 15 pounds pressure for 6 hours.

The vitamin B_1 used in these experiments was the preparation adopted by the League of Nations Health Organization, as described in the memorandum on the International Standard Preparation of Antineuritic Vitamin (B_1) (15).   It is commonly known as activated Java clay.   This preparation given daily in 15.0

mg. amounts contained sufficient vitamin B_1 to support adequate growth in rats of 40.0 to 50.0 gm. when vitamin B_2 G was present in the diet. Control experiments showed that the activated Java clay was devoid of vitamin B_2 G. Tests for the absence of vitamin B_1 and B_2 G in the basal ration were always made.

After weaning the rats were maintained on the basal vitamin B-free diet for a period of 2 weeks to insure the complete depletion of the body store of vitamin B_1 and B_2 G (15). They were placed in groups of five in round wire cages with raised bottoms to prevent coprophagy. They were fed once a day and the animals were allowed to eat as much as they desired. The animals were weighed weekly. At the beginning of the experiments the weights of the rats ranged from 40.0 to 50.0 gm.

The liver extract studied was the preparation made by Eli Lilly and Company; both the injectable and the powdered forms were used. The liver extract in the powdered form was dissolved in water and mixed with a small amount of food in a dish. The form prepared for parenteral use, when given by mouth, was mixed with food and given in the same way. All the rats cleared their dishes before more food was given. The animals receiving liver extract parenterally were given daily intraperitoneal injections.

The vegex was given in a 50 per cent aqueous solution. The amount administered daily in these experiments varied from 50.0 to 250.0 mg. When the daily doses were small, the test material was given individually with a pipette. Daily doses of 150.0 to 250.0 mg. were too large to be taken at one time. Accordingly, these amounts were given with the food and the animals watched until the dishes were empty.

The Vitamin B_1 and B_2 G Content of Powdered Liver Extract, Lilly No. 343

Experiment I.—Rats which had been kept for 2 weeks on the basal diet were divided into four groups. The basal diet was continued in all the rats. Those of the first group received in addition daily doses of 100 mg. of powdered liver extract, Lilly No. 343, an amount derived from 2.5 gm. of fresh whole liver. The diet of the rats of the second group was supplemented by the same dosage of liver extract and in addition 15.0 mg. of activated Java clay was given daily as a source of vitamin B_1. The animals of the third group received the basal diet plus the same amount of liver extract and in addition autoclaved bakers' yeast to supply vitamin B_2 G. The diet of the animals of the fourth group was supplemented by autoclaved bakers' yeast but contained no liver extract.

Group I.—Fig. 1, *a* shows the growth curves of four rats in the first group. These animals received the basal diet plus 100 mg. of liver extract daily. None

of these showed any appreciable gain in weight and all were dead by the end of the 2nd week. The results indicate that 100 mg. of powdered liver extract, Lilly 343, administered daily, do not contain sufficient vitamin B complex to support a normal growth rate in rats weighing 40 to 50 gm.

Group II.—Fig. 1, *b* shows the growth curves of four animals in the second group. These animals received the basal diet, supplemented by 100 mg. of liver extract daily and in addition 15 mg. of activated Java clay as a source of vitamin B_1. These curves are similar to those shown in Fig. 1, *a*. There was no appreciable gain in weight and all the animals died during the 2nd week. Since these animals received a sufficient amount of vitamin B_1 the results indicate that 100 mg. of powdered liver extract, administered daily, do not contain an adequate amount of vitamin B_2 G to support normal growth.
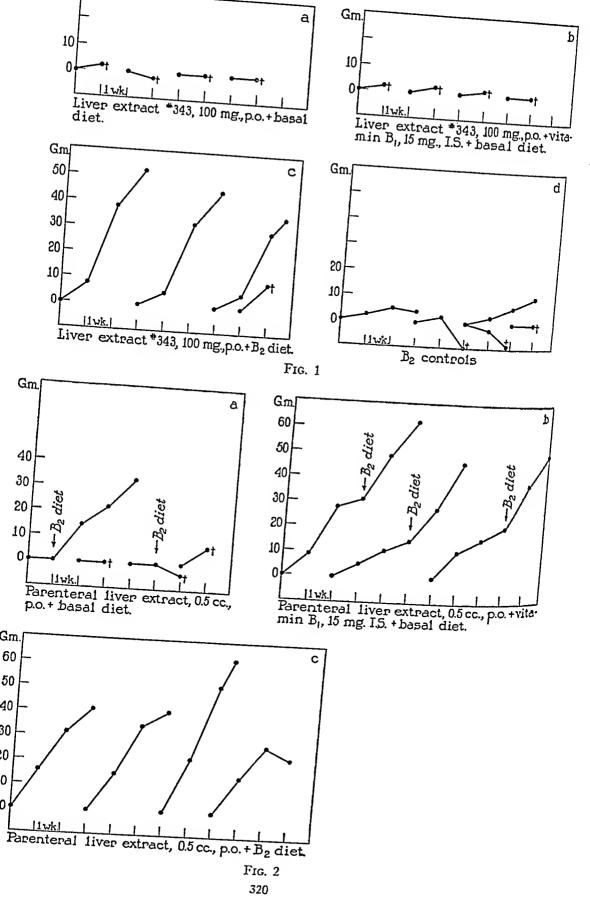
Group III.—Fig. 1, *c* presents the growth curves of animals of the third group. The animals of this group received the basal diet, supplemented by 100 mg. of liver extract daily and in addition an adequate amount of autoclaved bakers' yeast to supply vitamin B_2 G. Three of the four rats whose growth curves are presented maintained an average gain of over 10 gm. per week for a period of 3 weeks. The fourth animal died of intercurrent disease after gaining at the normal rate for the 1st week. These results demonstrate that 100 mg. of powdered liver extract when administered daily contain sufficient vitamin B_1 to maintain a normal rate of growth in rats weighing from 40 to 50 gm., provided that the animals receive an adequate supply of vitamin B_2 G in the diet.

Group IV.—Fig. 1, *d* presents the growth curves of the rats of the fourth group. These animals received the basal diet to which no liver extract was added. An adequate supply of vitamin B_2 G was supplied in the form of autoclaved bakers' yeast. This experiment served as a control of the vitamin B_1 content of the basal diet, supplemented by autoclaved yeast. Of these animals, two lived for 3 weeks, one gained 10 gm. in 2 weeks, the other 5 gm. in the same time. Three of the rats died during the 2nd or 3rd week without showing appreciable growth gains. These results indicate that the basal diet plus autoclaved bakers' yeast was deficient in vitamin B_1.

To summarize, the experimental results indicate that 100 mg. of powdered liver extract, Lilly No. 343, given daily to rats on a vitamin B-deficient diet contain an adequate amount of vitamin B_1 to maintain a normal growth rate, but that this amount of liver extract under similar conditions does not contain an adequate amount of vitamin B_2 G.

The Vitamin B_1 and B_2 G Content of Liver Extract (Lilly) in the Form Prepared for Parenteral Injection When Given Orally

Experiment II.—Rats which had been kept for 2 weeks on the basal diet were divided into three groups. The animals of the first group

FIG. 1

FIG. 2

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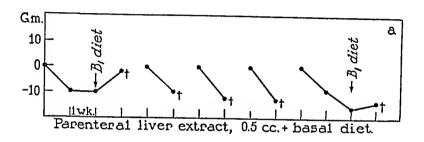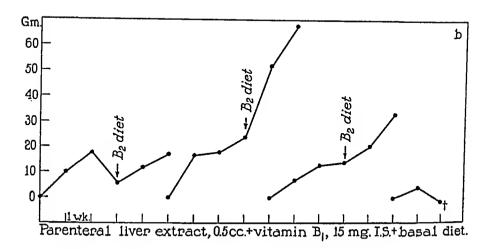
were given 0.5 cc. of injectable liver extract by mouth daily in addition to the basal diet. This amount of liver extract was equivalent to 2.5 gm. of fresh whole liver. The animals of the second group received the same basal diet and the same amount of liver extract. In addition each animal received 15 mg. of activated Java clay as a source of vitamin B_1. The animals of the third group received the basal diet and the same amount of liver extract. In addition an adequate amount of autoclaved bakers' yeast to supply vitamin B_2 G was supplied.
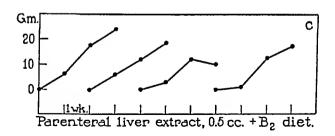
Group I.—In Fig. 2, *a* are presented the growth curves of the rats of the first group. These animals received the basal diet plus 0.5 cc. of liver extract in the form prepared for parenteral use. This was administered daily by mouth. By the end of the 2nd week two of the animals had died. At this time the diet of the two remaining animals was supplemented by autoclaved bakers' yeast to supply vitamin B_2 G. Hence, from this time on the diet was the same as that supplied to the animals of the third group. One of these rats died during the 3rd week. The other rat gained 33 gm. in 3 weeks after the addition of vitamin B_2 G. This was an average gain of 11 gm. per week. The results in the case of the first two animals and in the last two, before the diet was supplemented by vitamin B_2 G, indicate that 0.5 cc. of liver extract prepared for parenteral use but administered orally does not contain sufficient vitamin B complex to support normal growth. The results in the case of the second two animals after the diet was supplemented by vitamin B_2 G will be mentioned when considering the animals of the third group.
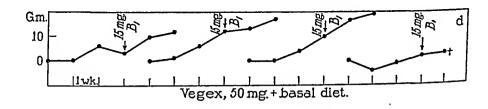
Group II.—In Fig. 2, *b* are presented the growth curves of the animals of the second group. These rats received the basal diet plus the same amount of liver extract as did the animals of the first group. In addition, 15 mg. of activated Java clay were given daily to each rat as a source of vitamin B_1. Of this group one rat gained an average of 10 gm. per week for the first 3 weeks. The second rat gained an average of 5 gm. per week, while the third averaged 7 gm. per week. These animals therefore gained in weight but not at a normal rate. At the end of the 3 week period, autoclaved bakers' yeast to supply vitamin B_2 was added to the diet. In the next 2 weeks each of the rats gained considerably over 10 gm. a week, a normal rate of growth. These results indicate that 0.5 cc. of liver extract in the form prepared for parenteral use when given daily by mouth does not contain an adequate amount of vitamin B_2 G to support normal growth, even though a sufficient amount of vitamin B_1 was added to the diet. However, when vitamin B_2 G was added, normal growth occurred.

Group III.—In Fig. 2, *c* are presented the growth curves of the rats of the third group. The animals of this group received the same amount of liver extract as did those of the first and second groups. In addition, these rats received the basal diet supplemented by an adequate amount of autoclaved bakers' yeast to
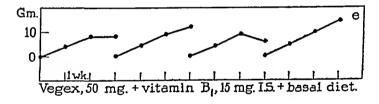
Fig. 3

supply vitamin B_2 G. Of this group, one rat gained in weight an average of 21 gm. per week. Two of the rats gained 13 gm. per week, while the fourth rat gained 8 gm. per week. Three of these animals therefore maintained a normal rate of growth. Also, one of the rats in Group I which received after the 2nd week a supplement of vitamin B_2 G in its diet gained 10 gm. a week, a normal rate of growth. These results indicate that 0.5 cc. of liver extract in the form prepared for parenteral use, when given daily by mouth, contains an adequate amount of vitamin B_1 to maintain a normal rate of growth, provided an adequate supply of vitamin B_2 G is present in the diet.

To summarize, this series of tests indicates that 0.5 cc. of liver extract in the form prepared for parenteral injection, when given daily by mouth to rats kept on a vitamin B-deficient diet, contains an adequate amount of vitamin B_1 to maintain a normal growth rate. Moreover, it appears that this amount of liver extract tested in the same way does not contain an adequate amount of vitamin B_2 G.

The Vitamin B_1 and B_2 Content of Liver Extract (Lilly), the Form Prepared for Parenteral Injection, When Given Parenterally

Experiment III.—Rats which had been kept for 2 weeks on the basal diet were divided into three groups. The animals of the first group received the basal diet and in addition, 0.5 cc. of liver extract daily by intraperitoneal injection. This amount of liver extract was derived from 2.5 gm. of fresh whole liver. The animals of the second group received the basal diet and the same amount of liver extract administered in the same way and in addition, 15.0 mg. of activated Java clay administered daily to each rat to supply vitamin B_1. The rats of the third group received the basal diet and daily injections of liver extract administered as to the animals of the other two groups and in addition, an adequate amount of autoclaved bakers' yeast to supply vitamin B_2 G.

Group I.—In Fig. 3, *a* are presented the growth curves of the animals of the first group. These rats were kept on the basal diet and received daily intraperitoneal injections of 0.5 cc. of liver extract. All of these rats lost weight and three of them died during the 2nd week. The two remaining rats at the beginning of the 3rd week were given daily 15 mg. of activated Java clay as a source of vitamin B_1. Thus, these two rats received the same diet as those of the second group. Both of these rats died during the 4th week. These results indicate that 0.5 cc. of liver extract in the form prepared for parenteral use and administered by intraperi-

toneal injection does not contain sufficient vitamin B complex to support normal growth. The results obtained after the addition of vitamin B_1 to the diet will be mentioned in the discussion of Group II.

Group II.—In Fig. 3, *b* are presented the growth curves of the animals of the second group. These rats received daily injections of liver extract, similar to those of the first group. The diet of the animals was the basal diet supplemented by 15 mg. of activated Java clay administered daily to supply vitamin B_1. Of the animals of this group, one rat died during the 3rd week, having shown no appreciable gain. A second rat averaged 3 gm. gain in weight per week, a third, 7 gm., and the fourth, 4 gm. Thus, none of these animals maintained normal growth over a period of 3 weeks. At the beginning of the 4th week the diet of the three remaining rats was supplemented with an adequate amount of auto-claved bakers' yeast to supply vitamin B_2 G. During the next 2 weeks one rat gained 8 gm. a week, another 10 gm., and a third, 22 gm. Hence, these rats maintained normal growth after the addition of vitamin B_2 G to the diet. These results and those of the two rats in the first group to which vitamin B_1 was given indicate that 0.5 cc. of liver extract in the form prepared for parenteral use when administered daily by intraperitoneal injection does not contain an adequate amount of vitamin B_2 G to support normal growth, even though a sufficient amount of vitamin B_1 was added to the diet. The results which followed the addition of vitamin B_2 to the diet will be mentioned in the third group.

Group III.—In Fig. 3, *c* are presented the growth curves of the animals of the third group. The rats of this group received the basal diet and injections of liver extract similar to those of the two preceding groups. In addition, the diet was supplemented by an adequate amount of autoclaved bakers' yeast to supply vitamin B_2 G. Of the animals of this group, one rat made an average weekly gain of 8 gm., another of 6 gm., a third of 5 gm., and the fourth rat of 3 gm. Thus, none of these animals grew at a normal rate. The animals of the second group of this series maintained normal growth after the addition of vitamin B_2 G to the diet. However, these rats were receiving daily 15 mg. of activated Java clay as a source of vitamin B_1. The results of the animals of the third group of Experiment II indicate that 0.5 cc. of liver extract in the form prepared for parenteral use, when administered by mouth, contains sufficient vitamin B_1 to support normal growth. However, the animals of this, the third group of Experiment III, did not grow at a normal rate when the liver extract was given by intraperitoneal injections. It therefore seems clear that the vitamin B_1 present in the liver extract was not as effective in supporting growth when given parenterally as when a similar amount of the same preparation is given by mouth.

To summarize, this series of tests indicates that 0.5 cc. of liver extract in the form prepared for parenteral use when administered daily by intraperitoneal injection to rats kept on a vitamin B-deficient diet does not contain an adequate amount of vitamin B_2 G to support

normal growth. Moreover, it appears that the amount of vitamin B_1 present in liver extract of this form is not so effective in supporting normal growth when given by intraperitoneal injection as it is when given by mouth.

The Vitamin B_1 and B_2 G Content of Vegex

Experiment IV.—Rats which had been kept for 2 weeks on the basal diet were divided into five groups. The animals of the first group received the basal diet and in addition, 50 mg. of vegex administered daily to each rat. The animals of the second group received a similar amount of vegex as those of the first group. In addition, they received the basal diet supplemented by 15 mg. of activated Java clay daily as a source of vitamin B_1. The animals of the third group received a similar daily amount of vegex as those of the first and second groups. In addition, these animals received the basal diet supplemented by an adequate amount of autoclaved bakers' yeast to supply vitamin B_2 G. The animals of the fourth group received the basal diet and in addition, 150 mg. of vegex administered daily to each rat. The animals of the fifth group received the basal diet and in addition, 250 mg. of vegex administered daily to each rat.

Group I.—In Fig. 3, *d* are presented the growth curves of the rats of the first group. These animals received the basal diet supplemented by 50 mg. of vegex administered daily to each rat. During the first 3 weeks the greatest average individual gain in weight was 4 gm. The other animals of this group averaged 2 to 3 gm. growth gain per week. At the end of the 3rd week the diet was supplemented with 15 mg. of activated Java clay as a source of vitamin B_1. The growth gains following this addition were negligible. Hence, after the 3rd week these rats received the same diet as those of Group II. The results obtained after this addition will be mentioned in the discussion of the second group. The results obtained during the first 3 weeks of this experiment indicate that 50 mg. of vegex given daily to rats kept on the basal diet do not contain sufficient vitamin B complex to support normal growth.

Group II.—In Fig. 3, *e* are presented the growth curves of the animals of the second group. These rats were fed the basal diet supplemented by 50 mg. of vegex daily, and in addition, 15 mg. of activated Java clay as a source of vitamin B_1. During the 3 week period the greatest average weekly gain for any of the rats was 4 gm. per week. The others averaged 2 to 3 gm. per week. Similarly, the average weekly gain of the rats of the first group after vitamin B_1 was added to the diet was negligible. Thus, a normal rate of growth was not maintained by any of these

Vegex, 50 mg. + B₂ diet.

Vegex, 150 mg. + basal diet.

Vegex, 250 mg. + basal diet.
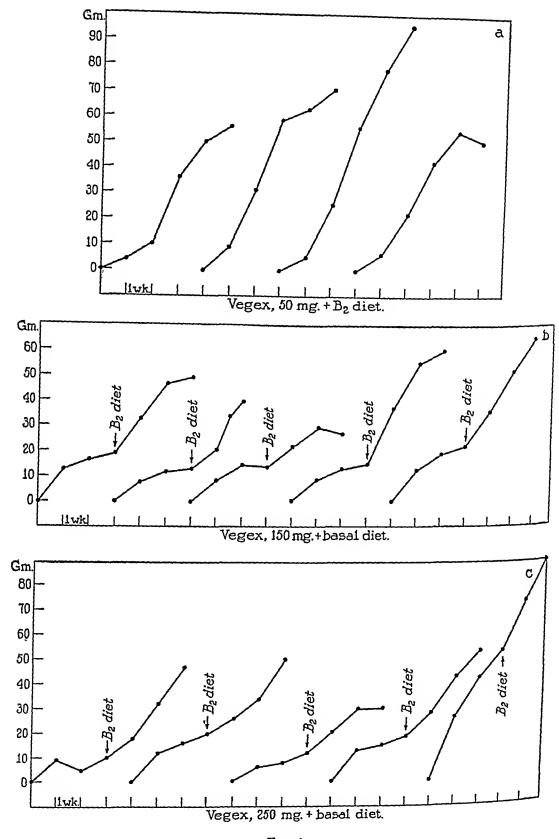
Fig. 4

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rats. These results indicate that 50 mg. of vegex administered daily to rats kept on the basal diet, supplemented by an adequate amount of vitamin B_1, do not contain a sufficient amount of vitamin B_2 G to support normal growth.

Group III.—In Fig. 4, *a* are presented the growth curves of the animals of the third group. These rats were fed the basal diet supplemented by 50 mg. of vegex daily, and in addition, an adequate amount of autoclaved bakers' yeast as a source of vitamin B_2 G. All of these rats for a period of 5 weeks made an average gain in weight of over 10 gm. per week; one rat averaged 19 gm. per week. A normal rate of growth occurred in all instances. These results indicate that 50 mg. of vegex administered daily to rats kept on the basal diet supplemented by an adequate amount of vitamin B_2 G contain sufficient vitamin B_1 to support normal growth.

Group IV.—In Fig. 4, *b* are presented the growth curves of the rats of the fourth group. These animals received the basal diet supplemented by 150 mg. of vegex administered daily. None of these animals grew at a normal rate for the first 3 weeks. One rat averaged 7 gm., the others, 4 to 6 gm. per week. At the end of the 3 week period the diet was supplemented by an adequate amount of autoclaved bakers' yeast to supply vitamin B_2 G. Following this addition of vitamin B_2 G to the diet, four of the five rats gained over 10 gm. per week, thus maintaining a normal rate of growth. These results indicate that 150 mg. of vegex given daily to rats maintained on the basal diet do not contain an adequate amount of the vitamin B complex to support normal growth. However, a normal rate of growth is obtained under similar conditions when a sufficient amount of vitamin B_2 G is added to the diet. Hence, it appears that 150 mg. of vegex, under the conditions described, is adequate in its content of vitamin B_1 but deficient in its content of vitamin B_2 G to support normal growth

Group V.—In Fig. 4, *c* are presented the growth curves of the rats of the fifth group. These animals received the basal diet, supplemented by 250 mg. of vegex administered daily. Of these rats, only one gained at a normal rate for the first 3 weeks. The other four rats averaged 3 to 6 gm. gain in weight per week. At the end of this 3 week period the diet was supplemented by an adequate amount of autoclaved bakers' yeast to supply vitamin B_2 G. After this addition of vitamin B_2 G to the diet three of the five rats gained at a normal rate. These results indicate that 250 mg. of vegex administered daily to rats kept on the basal diet do not contain sufficient vitamin B complex to support normal growth. However, a normal rate of growth is obtained under similar conditions, provided vitamin B_2 G is added to the diet. Hence, 250 mg. of vegex under the conditions stated is adequate in its content of vitamin B_1 but deficient in its content of vitamin B_2 G.

To summarize, these results indicate that 50, 150, and 250 mg. of vegex administered daily to rats on a vitamin B-deficient diet contain an adequate amount of vitamin B_1 to maintain a normal rate of growth, but that these amounts of vegex under similar conditions do not contain an adequate amount of vitamin B_2 G.

DISCUSSION

The rat growth curves presented in this communication do not include those of all the experimental animals. The ones presented were selected as typical. Relatively large numbers of rats were used in all the experiments and the results were entirely consistent with those presented. Furthermore, the experiments were repeated as a whole. The results obtained in the second group of experiments were entirely in keeping with those obtained in the first.

From the experiments presented, it can be concluded that 100.0 mg. of powdered liver extract, Lilly No. 343, and 0.5 cc. of the form prepared for parenteral use, when given daily by mouth to rats of 40.0 to 50.0 gm. weight, on a basal vitamin B-free diet, do not contain enough vitamin B complex to support normal growth. Furthermore, when vitamin B_1 is added to the diet by the daily administration of 15.0 mg. activated Java clay, an amount more than sufficient to give adequate growth to such rats if B_2 G in an adequate amount were present in the diet, there is no appreciable growth gain. However, when autoclaved bakers' yeast as a source of vitamin B_2 G is substituted for the vitamin B_1, a normal rate of growth occurs. Hence, it seems clear that these two forms of liver extract when given by mouth under the conditions described contain adequate vitamin B_1 to support normal growth, but are deficient in vitamin B_2 G. This is not in accordance with the results reported by Guha (9–11) who obtained normal growth of rats under similar conditions with 40.0 to 60.0 mg. daily of the same liver extract preparation. Guha used rats fed a diet containing a vitamin B_1 concentrate. He stated that this concentrate contained some vitamin B_2 G, but not enough to support growth. It is possible that the vitamin B_1 concentrate used by Guha might have contained enough vitamin B_2 G to support normal growth when added to the small amount present in liver extract. Guha did not attempt to assay the liver extract for vitamin B_1 as he did not obtain growth with as much as 120.0 mg. when the vitamin B_1 concentrate was omitted from the diet. He concluded that the reason for the failure to obtain growth was the low vitamin B_1 content of liver extract. Our experiments demonstrate that the failure with 100.0 mg. of liver extract is due to its low vitamin B_2 G content.

The experiments in which liver extract was given by intraperitoneal

injection demonstrate that liver extract in the form prepared for parenteral use does not contain a sufficient amount of the vitamin B complex to support normal growth of rats under the experimental conditions observed. The amount of liver extract injected was the same as was tested by the oral route in other experiments. It may be recalled that normal growth was not obtained in the case of rats which received daily intraperitoneal injections of liver extract and a diet supplemented by autoclaved bakers' yeast as a source of vitamin B_2 G. Since the same preparation of liver extract was shown to contain an adequate amount of vitamin B_1 when given by mouth in the same dosage, it appears that the vitamin B_1 present in this form of liver extract is not as effective when given by intraperitoneal injection as it is when administered orally.

The experiments employing vegex, a brewers' yeast concentrate, as a source of water-soluble vitamin indicate that it contains a large amount of vitamin B_1 but a relatively small amount of vitamin B_2 G. Subnormal growth was obtained when 50, 150, and 250 mg. of vegex were given daily to rats on a basal vitamin B-free diet. However, when vitamin B_2 G was added to the diet this growth was normal. Strauss and Castle (2) in the treatment of pernicious anemia used 12 gm. of vegex with 150 cc. of normal human gastric juice in an incubated digest. This amount, given daily over a period of 10 days, produced typical remissions in patients with pernicious anemia. 50 mg. of vegex, given daily to a rat of 50 gm. weight is comparable to a daily dosage of 50 gm. in a person weighing 50 kilos, if calculated by weight. Since 50 mg. of vegex were shown to be inadequate in vitamin B_2 G to support growth of rats, and since a much smaller amount administered to human beings on a weight for weight basis is adequate in content of the extrinsic anti-anemic factor, evidence is at hand that the growth-promoting factor and the extrinsic anti-anemic factor are dissimilar. Although the inability to support growth in rats appears to be evidence of the inadequate content of vitamin B_2 G in a particular foodstuff, it should be borne in mind that the term vitamin B_2 G is a general one referring to a thermostable accessory food factor. The commonly accepted criterion for the presence of this factor in foodstuffs is the ability of the material in question to promote normal growth in rats. The absence of this quality in a substance such as

vegex, known to possess another biological property, the ability to promote hematopoiesis, indicates the necessity of further study of the various components of the heat-stable accessory food factor, vitamin B$_2$ G.

<div style="text-align: center;">CONCLUSIONS</div>

1. Liver extract powder, No. 343 Lilly, and the same material prepared for parenteral use, when administered daily by mouth in amounts derived from 2.5 gm. of fresh whole liver, to rats weighing from 40 to 50 gm., contain sufficient vitamin B$_1$ to support normal growth, provided the animals receive in addition an adequate amount of vitamin B$_2$ G. Moreover, liver extract in the forms mentioned, administered in the same amounts, does not contain sufficient vitamin B$_2$ G to maintain normal growth of similar rate when all other necessary constituents of the diet are provided.

2. Liver extract (Lilly) in the form prepared for parenteral use, when administered daily by intraperitoneal injections, in amounts derived from 2.5 gm. of fresh whole liver, to rats under standard experimental conditions, does not contain sufficient vitamin B$_2$ G to maintain normal growth. Furthermore, the amount of vitamin B$_1$ present in liver extract in this form is not as effective in supporting normal growth when given by intraperitoneal injection as it is when given by mouth.

3. Vegex, when administered daily in amounts of 50, 150, and 250 mg. to rats of 40 to 50 gm. in weight contains sufficient vitamin B$_1$ to maintain normal growth of the rats, provided the animals receive in addition an adequate amount of vitamin B$_2$ G. However, vegex in the same amounts does not contain sufficient vitamin B$_2$ G to support normal growth of similar rats when all other necessary constituents of the diet are provided.

4. These experiments indicate that the extrinsic, anti-anemic factor of Castle and the thermostable growth-promoting food constituent, commonly known as vitamin B$_2$ G, are not identical.

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THE EFFECT OF HEMOGLOBIN INJECTIONS ON ERYTHROPOIESIS AND ERYTHROCYTE SIZE IN RABBITS RENDERED ANEMIC BY BLEEDING

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PLATES 21 AND 22

Variations in the size of red blood cells and in their hemoglobin content are striking and important features in various types of anemia in human beings. In many instances these variations are sufficiently distinctive to be of importance in diagnosis and treatment. In certain types of anemia the red cell is small and its hemoglobin content is less than normal, whereas in others the erythrocyte is large and contains more than the normal amount of pigment. Furthermore, during the treatment of macrocytic anemia, the average cell may change during therapy from a macrocyte to one smaller than normal, and the hemoglobin content of the cell instead of being greater may become less than normal. These variations indicate the necessity of an alteration of therapy, involving a shift from one stimulating to cell stroma formation to one productive of an increase in blood pigment (1). The bone marrow changes during these cellular alterations have been studied in pernicious anemia by Peabody (2), and in sprue anemia by Rhoads and Castle (3). Very little is known of the actual mechanism involved in the alterations of cell size. It has seemed important to study in greater detail the production of such alterations. The method chosen was to increase the available body supply of blood pigment in animals rendered anemic by bleeding.

It is frequently stated that blood regeneration takes place less rapidly after external hemorrhage than after destruction of red blood corpuscles has occurred within the body. This observation has been confirmed experimentally by the work of McMaster and Haessler (4). They produced an anemia in rabbits and observed that the hemoglobin loss was replaced more rapidly if the animals were given subcutaneous

injections of hemoglobin. They also demonstrated an extensive increase in the hematopoietic portion of the bone marrow in these animals. They concluded that the factor which determines the increase in amount of the red bone marrow during anemia is the presence in the body of hemoglobin or its precursor in an amount greater than that which can be utilized by the existing active marrow. Whipple and his coworkers (5) studied dogs with chronic severe posthemorrhagic anemia. They found that if a stroma-free solution of hemoglobin were administered intravenously or intraperitoneally to these dogs, 80 to 90 per cent of the hemoglobin injected was recovered in newly formed red cells. In neither of these experiments were observations of changes in cell size or in cell hemoglobin content reported.

The present paper is a study of the erythrocytes of the peripheral blood and of erythropoiesis in rabbits rendered anemic by repeated bleeding. Certain of the animals were treated with subcutaneous injections of a stroma-free hemoglobin solution. Particular attention was paid to the size and hemoglobin content of the red blood cells. The marrow alterations in these animals were also studied in detail with a view to explaining, if possible, the mechanism operative in bringing about the observed changes in the cells of the peripheral blood.

Methods

Twenty rabbits were rendered anemic by almost daily bleedings by the method of cardiac puncture. The hemoglobin level in all the animals was brought as near to 50 per cent as was possible. This could be done only by varying the amount of blood removed and the frequency of the bleedings in the individual animals. The rabbits were divided into two groups. Subcutaneous injections of a stroma-free hemoglobin solution were given daily to the animals of the first group. Animals of the second group received no hemoglobin injections and served as controls. This hemoglobin solution was prepared from rabbit blood by the method of Sellards and Minot (6). The amount of hemoglobin injected was always in excess of the amount present in the blood removed by cardiac puncture. Hence this injection was regarded not only as a matter of replacement of hemoglobin removed, but also as a means of increasing the amount of available blood pigments. The content of hemoglobin in the solution injected varied from 120 to 140 per cent as determined by the Sahli acid hematin method. The amounts injected each day varied from 15 to 25 cc., depending on the amount of blood removed on that day. If no bleeding was performed, nevertheless 15 cc. of hemoglobin solution were injected.

Erythrocyte counts, hemoglobin determination, and measurements of the mean corpuscular volume were made on alternate days on all the rabbits. The red cells were counted by the usual method using pipettes certified for accuracy by the United States Bureau of Standards. The hemoglobin was determined by the Sahli acid hematin method using calibrated tubes and standards. Determinations of the mean corpuscular volume (M. C. V.) were made with the Wintrobe hematocrit tube (7). From data so obtained the color index and the M. C. V. were calculated.

It was possible to continue the experiment as projected for approximately 6 weeks. Several of the rabbits of each group had lived for 9 weeks when the experiment was terminated. Necropsies were performed on all the animals. Microscopical studies of the femoral bone marrow were made on tissue fixed in Zenker's fluid with acetic acid. The sections were stained with eosin and methylene blue.
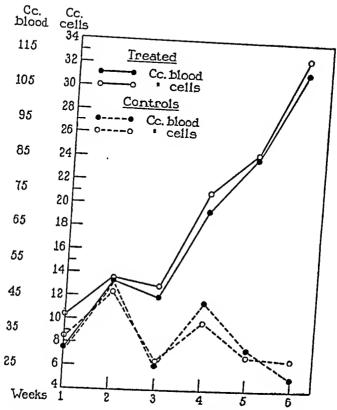
<center>EXPERIMENTAL</center>

Maintenance of Anemia

In Text-fig. 1 are presented for both groups of animals the average total volumes of blood and of cells removed weekly per animal. During the first 2 weeks approximately equal volumes of blood and cells were removed from all the animals. In order to maintain the hemoglobin level at about 50 per cent it was necessary after the 2nd week to increase the amount of blood removed from the treated animals. During the 3rd week for the treated series the average total amount of blood removed per animal was 45 cc., during the 4th week 70 cc., the 5th week 85 cc., and the 6th week 110 cc. Thus the average total amount of blood removed over the period of 6 weeks from each of the treated animals was 390 cc. The average total amount of cells removed closely paralleled the amount of blood withdrawn. In order to maintain the level of hemoglobin of the control animals at about 50 per cent, it was not necessary to subject them to such a great blood loss. Consequently, the average total volume of blood removed weekly from each of the control animals fell sharply after the 2nd week. During the 3rd week this amount was 25 cc., for the 4th week 43 cc., the 5th week 30 cc., and the 6th week 26 cc. Thus the average total volume of blood removed over a period of 6 weeks from each control animal was 204 cc. The average total volume of cells closely paralleled the volume of blood removed.

These figures show that in order to maintain a similar grade of

anemia in the animals of the two groups, it was necessary to subject the treated animals to a much greater loss of blood than was required in the control group. It seems clear, therefore, that in the treated animals erythropoiesis proceeded at a much more rapid rate than in the controls.
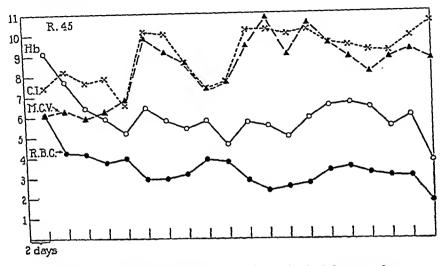
TEXT-FIG. 1. The average total volumes of blood and of cells removed weekly per animal.
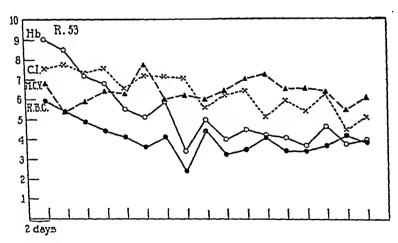
Alterations of the Erythrocytes in the Animals Receiving Hemoglobin, and in the Control Animals

In Text-fig. 2 are presented the blood studies of an animal considered to be typical of the group which received daily injections of hemoglobin. A total of 495 cc. of blood was removed from this animal over a period of 44 days. During that time a total of 596 cc. of a stroma-free hemoglobin solution was administered by subcutaneous injection. On the 44th day accidental death occurred.

At the beginning of the experiment the blood levels of this animal were as follows: R. B. C. 6,100,000, hemoglobin 91 per cent, M. C. V.

TEXT-FIG. 2. The blood studies of a typical animal of the treated group.

TEXT-FIG. 3. The blood studies of a typical animal of the control group.

0.610, color index 0.740. During the first 10 days of the experiment the red count and hemoglobin levels fell abruptly while the M. C. V.

and color index did not vary appreciably. However, during the 2nd week the color index rose to 1.01 and the M. C. V. to 0.980. It was difficult to maintain the blood of this rabbit at a hemoglobin level of 50 per cent. The anemia obtained during the experiment was represented by an average erythrocyte level of 3,000,000 and a hemoglobin content of 55 per cent. The mean corpuscular volume and color index were consequently higher after the first 10 days than they were at the beginning of the experiment. At the death of the animal the blood levels were: R. B. C. 1,850,000, hemoglobin 38 per cent, M. C. V. 0.870, color index 1.03.

The results of the blood studies of a control animal are presented in Text-fig. 3. A total of 235 cc. of blood was removed by cardiac puncture from this animal over a period of 42 days. This animal received no hemoglobin solution and accidental death occurred on the 42nd day. The blood levels at the beginning of the experiment were: R. B. C. 5,950,000, hemoglobin 90 per cent, M. C. V. 0.675, color index 0.755. Thus the blood elements of this control animal and those of the treated one presented above were at similar levels at the outset. As in the case of the treated animal the red count and hemoglobin of this rabbit dropped rapidly during the first 10 days, whereas the M. C. V. and color index did not change appreciably. On the 10th day the blood elements of the two animals were at approximately equal levels. However, after the 2nd week the color index and M. C. V. of the control animal fell gradually. During the remainder of the experiment the blood levels were considerably lower than they were at the beginning. The average hemoglobin level was 45 per cent and the erythrocyte count 3,500,000. At the death of the animal the blood levels were: R. B. C. 3,900,000, hemoglobin 40 per cent, M. C. V. 0.610, color index 0.512.

Thus in the treated animal an anemia was produced in which the size of the red blood cell and the color index were greater than normal, whereas in the control animal an anemia was produced in which the size of the red blood cell and the color index were less than normal.

The weekly average blood studies of the animals of both groups are presented in Table I. At the beginning of the experiment the average red cell counts and hemoglobin levels of the treated animals were higher than those of the control animals. However, the mean corpus-

cular volumes and the color indices of both groups of animals were approximately the same. During the course of the experiment the average hemoglobin levels of the treated animals were consistently higher than those of the controls. The lowest average hemoglobin level of the former group was 53 per cent, while that of the latter group was 42 per cent. This dissimilarity of the hemoglobin levels of the two groups of animals was due to the great difficulty in maintaining the desired grade of anemia of the treated animals, although they were subjected to a blood loss which was far greater than that of the control animals. The average erythrocyte counts of all animals were approximately equal until the 4th week. After that time the average

TABLE I
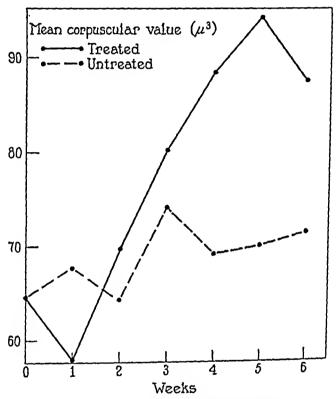
Combined Averages of Blood Studies for Each Week

Weeks	R.B.C. in injected animals	R.B.C. in control animals	Hemo-globin in injected animals	Hemo-globin in control animals	M.C.V. in injected animals	M.C.V. in control animals	Color index in injected animals	Color index in control animals
	millions	millions	per cent	per cent	$c. \mu$	$c. \mu$		
0	5.76	4.69	91	72	0.645	0.645	0.798	0.771
1	5.38	3.98	90	59	0.580	0.676	0.802	0.754
2	3.82	3.72	53	48	0.697	0.643	0.808	0.666
3	4.03	3.66	59	43	0.802	0.741	0.756	0.745
4	3.45	3.53	56	43	0.884	0.681	0.858	0.622
5	3.01	3.80	55	52	0.941	0.706	0.917	0.683
6	3.37	3.54	55	42	0.875	0.714	0.830	0.654

red cell counts of the treated rabbits were lower than those of the control animals and so remained throughout the experiment. Hence, during the latter part of the study the experimental anemia in the animals which received hemoglobin was characterized by lower red cell counts and higher hemoglobin levels than were found in the anemia of the control animals.

The average mean corpuscular volumes of the two groups of animals are presented for each week of the experiment in Text-fig. 4, and in Table I. At the beginning of the experiment the average mean corpuscular volumes of each group of animals was 0.645. During the 2nd week the average size of the cell of the treated animals began to increase. This increase in cell size continued until the 5th week when

it had reached a level of 0.941. During the 5th week it fell to 0.875. Meanwhile, the average mean corpuscular volume of the control animals increased very little throughout the course of the study. The highest level obtained in these animals was 0.741 at the 3rd week. However, it must be pointed out that there was an actual increase in the average cell size of the untreated rabbits. At the 6th week the average increase in the size of the red blood cell of the treated animals
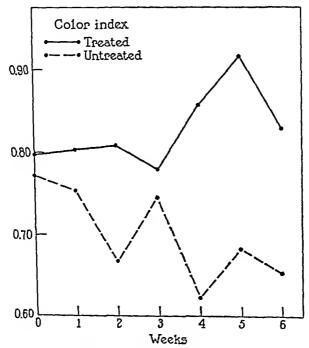
TEXT-FIG. 4. The average mean corpuscular volumes of both groups of animals.

was 36 per cent, while that of the control animals was 10 per cent. Thus there was a steady increase in the size of the erythrocytes of all the animals, although it was much greater in those which received injections of hemoglobin.

The average color indices of the two groups of animals for each week are presented in Text-fig. 5 and Table I. At the beginning of the experiment the average color index of the treated animals was 0.798.

There was no appreciable change in the color index of these rabbits until after the 3rd week, when it increased steadily until it reached its highest level at the 5th week.   This level was 0.917.   At the end of the experiment the average color index of these animals was 0.830. In contrast to these results are the levels of the color indices of the control animals.   At the beginning of the experiment the average color index of these animals was 0.771.   During the entire experiment

TEXT-FIG. 5. The average color indices of both groups of animals.

the average color indices of this group were always lower than the original levels.   At the termination of the experiment the average color index was 0.654.   Thus, whereas an average increase of 4 per cent occurred in the color indices of the treated animals, a decrease of 15 per cent occurred in those of the control animals.

To summarize: In the treated animals an anemia was produced in which the average cell size and the average color index were higher

than were the original levels. The anemia in the control animals on the other hand was marked by an average cell size and color index strikingly lower than were those of the treated animals. Also in the treated animals the erythrocyte counts were lower and the hemoglobin levels were higher than those of the control animals. This occurred in spite of the much greater loss of blood to which the treated animals were subjected.

Alterations in the Marrows of Hemoglobin Replacement and Non-Replacement Animals

The femoral marrow of the normal rabbit is a reddish gray in color. Microscopically it is very fatty and fairly acellular (Fig. 1). On higher magnification, it is seen to contain large fat spaces surrounded by cellular elements which for the most part are adult erythrocytes and normoblasts, with a limited number of young undifferentiated cells. White blood cells are present, the predominating cell of this series being the eosinophil and the eosinophilic myelocyte.

The marrows of the control rabbits were redder in the gross, and histologically more active than were those of normal animals. Fat spaces were still numerous, but there was a moderate increase in cellularity (Fig. 2). Higher magnification showed many nucleated red cells or normoblasts. There were numerous cells ordinarily classed as erythroblasts because of their basophilic cytoplasm and nuclei containing fairly heavy masses of chromatin. There were more leukocytes than are seen in the marrows of normal animals. Myelocytes were fairly numerous. The blood spaces were open and contained mature erythrocytes. The distinguishing features between these marrows and the normal were: (1) fewer fat spaces, (2) many more normoblasts, (3) numerous erythroblasts, (4) white cell hyperplasia.

In brief, the marrows of the control animals were more active than normal, the hyperplasia being normoblastic and erythroblastic accompanied by an increased number of myelocytes.

The femoral marrows of the animals receiving hemoglobin presented a striking contrast to those just described (Fig. 3). They were deep red in color, solid, and extremely cellular. The venous sinusoids were narrow and compressed. The spaces normally filled with fat were almost entirely replaced by collections of large cells with basophilic

cytoplasm. Very few mature erythrocytes were present. By high magnification, the predominating cell was seen to be one with a moderately basophilic cytoplasm of somewhat variable contour and size (Fig. 4). The nuclei were large and were vesicular, with a loosely arranged network of chromatin. Mitotic figures were occasionally seen. These cells were considered to be late erythroblasts or megaloblasts, according to the classification of Peabody (2). Numerous erythroblasts were present. Normoblasts were fewer than in the control marrows. A moderate hyperplasia of cells of the myelocytic series was apparent.

The most striking features differentiating these marrows from those of the controls were (1) the solid packing of cells leaving very few fat spaces, (2) the relative scarcity of adult erythrocytes, (3) the relative decrease in numbers of normoblasts, (4) the frequent occurrence of megaloblasts, (5) the presence of mitotic figures.

It may be pointed out that the marrows of the rabbits which received the stroma-free hemoglobin solution were similar in some respects to those of patients with pernicious anemia. They were hyperplastic, with erythropoiesis predominant at the earlier stages as evidenced by the frequent occurrence of megaloblasts and erythroblasts. The activity of the marrows of the control animals was similar to that seen in chronic anemias associated with loss of blood. The predominant cells were the normoblast and late erythroblast.

DISCUSSION

As pointed out by McMaster and Haessler (4), one striking feature of the experimental results is the enormously increased hematopoiesis in the treated animals over that seen in the control group. Since the material used in treatment was a hemoglobin solution which had been freed from stroma, no known source of new cell framework was supplied. These facts indicate that the animal body possesses a large reserve of stroma-building material under the experimental conditions observed. In spite of the greater withdrawal of blood, the animals in the treated series maintained a blood hemoglobin level higher than that of the controls. The erythrocyte count, on the other hand, tended to be lower than in the controls. This disproportion between hemoglobin values and numbers of erythrocytes resulted in an increase of the color index and was paralleled by an increase in the mean cor-

puscular volume of the erythrocytes. Since the average cell size was greater in the treated animals, the increased hemoglobin content of the cell was not due to a concentration of pigment, but to a greater amount of pigment-containing cell stroma. This phenomenon of increased cell size and hemoglobin content is seen in the so called macrocytic anemia of sprue and pernicious anemia. In these conditions an excess of pigment, bilirubin, is present in the blood stream. It disappears when effective therapy has been instituted and improvement in blood values established. It would appear that in the clinical macrocytic anemias the function of erythrocyte stroma formation is specifically exhausted and pigment metabolism not interfered with. Hence, in an endeavor to meet the metabolic demand for oxygen, a larger cell is formed containing more hemoglobin than normal. In the experiments described, an analogous situation has been established by attempting to exhaust the cell stroma-building material of the body and supplying pigment in excess. Changes in size and hemoglobin content of the erythrocytes analogous to those found in macrocytic anemia in human beings were induced.

In the microcytic anemias of human beings, relieved by iron and in part due to an iron deficiency, the cell size is small and its content of hemoglobin low. It is suggested that the causal mechanism of these changes is the reverse of that just discussed. Here cell stroma formation is relatively uninhibited and pigment supply is lacking. In an endeavor by the body to compensate, a large number of cells are formed which contain relatively little hemoglobin. Here again, a somewhat analogous condition developed in the experimental animals which were untreated. The color index fell during the experiment. The mean corpuscular volume did not decrease proportionately. This apparent discrepancy may be explained, in part at least, by the persistently moderately elevated reticulocyte counts obtained in both groups of animals.

No essential difference in the rate of reticulocyte production was observed in the two groups of animals. From that observation it is clear that the difference in erythrocyte size seen in the two groups was not due to a variation in the rate of reticulocyte production.

The difference between the histological picture of the bone marrows of the two experimental groups was most striking. The greater pro-

portion of erythroblasts and megaloblasts seen in the marrows of the treated animals was similar in some respects to the marrow change in the macrocytic anemia of human beings. This suggests that under circumstances of lack of stroma-forming material, as in the macrocytic anemias of human beings or of excessive drain on stroma formation as in the experiments described, the marrow tends to assume a younger cell type. The objection may be raised that the difference in the rate of erythropoiesis explained the variations in the histological pictures of the marrows of the two groups. It must be recalled that the difference in hematopoietic rate was due to treatment with hemoglobin plus the stimulus of anemia. The marrow histology in the untreated animals was of a type seen in anemia in human beings following prolonged loss of blood. Though hematopoietic activity was present, the cell type was different and furthermore the degree of cellular hyperplasia was enormously greater in the treated group.

SUMMARY

1. An anemia characterized by increased size of the red blood cells and a high color index was produced in rabbits by repeated bleeding and by the subcutaneous injection of stroma-free hemoglobin solution.

2. The bone marrow of these rabbits reverted to a more primitive stage than did the marrows of rabbits rendered anemic in the same manner but not treated with hemoglobin.

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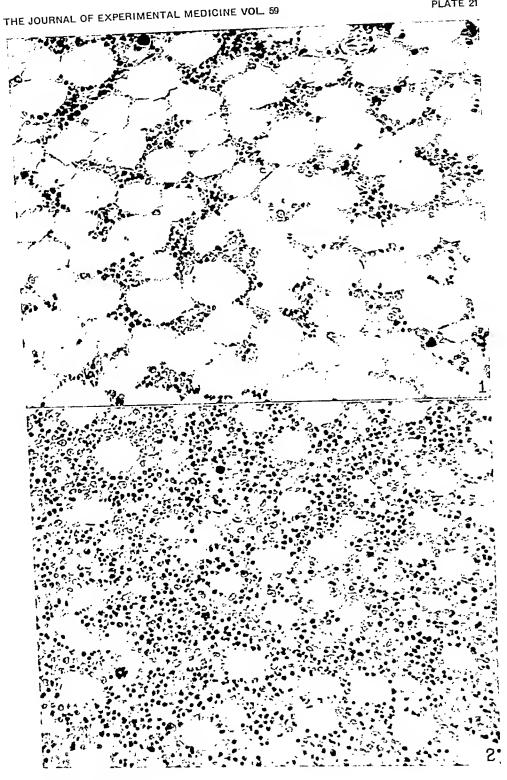
EXPLANATION OF PLATES

PLATE 21

FIG. 1. The femoral bone marrow of a normal rabbit showing numerous fat spaces and a fairly acellular marrow. × 300.

FIG. 2. The femoral bone marrow of a control rabbit showing relatively few fat spaces and many normoblasts. × 300.

PLATE 22

FIG. 3. The femoral bone marrow of a treated rabbit showing a very cellular tissue with absence of fat spaces. × 300.

FIG. 4. The same marrow as Fig. 3, under higher power with very few normoblasts and several megaloblasts. × 1000.

PLATE 21

(Miller and Rhoads: Erythropoiesis and erythrocyte size)

STUDIES ON EXPERIMENTAL HYPERTENSION

I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia[*][†]

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PLATES 23 AND 24

The production of elevated blood pressure in animals has been attempted (1–16) by various methods involving injury to the kidneys. In the experiments of long duration (7–16), for the purpose of producing persistent hypertension, the methods used were injection of nephrotoxic substances (7), irradiation of the kidneys by Roentgen rays (8), renal venous stasis (9–11) and excision of varying amounts of kidney tissue, with or without ligation of some branches of the renal arteries (12–16). The elevation of blood pressure which occurred as a result of some of these methods did not prove persistent. Cash (15), one of those who used the method mentioned last, drew attention to the fact that the increase of pressure occurred only when some necrotic kidney tissue was undergoing absorption within the body. He gave as the conditions under which he observed temporary elevation of blood pressure in dogs, that the total kidney substance be reduced at least 50 per cent and that, in addition a portion of kidney which has been deprived of its circulation be allowed to remain *in situ*.

Although it has been suggested (Fahr (17)) that renal ischemia, by itself, may play an important part in the development of the hypertension which is associated with more or less diffuse vascular disease in man, yet, up to the present time, the validity of this contention has

not been investigated experimentally in animals by a study desi[g]
to test the effect on blood pressure of renal ischemia alone.

In the investigation here reported the working hypothesis ado[p]
was that ischemia *limited to the kidneys* may be the initial conditi[o]
the pathogenesis of the hypertension that is associated with nep[h]
sclerosis.   If this be true, then renal ischemia, no matter how
duced, should be followed by elevation of blood pressure.   This re[p]
deals with the effect on the blood pressure of dogs of experiment[al]
produced ischemia limited to the kidneys.   The simplest method
this purpose being obviously constriction of the main renal arte[ry]
this was the method chosen.

Method

Animals and Diet.—Dogs of various mixed breeds were used.   The animals
females, varied in weight and age.   Their exact age was not known but they w[ere]
all full grown, seemingly normal dogs.   Examination of blood pressure, blood [and]
urine during a long control period revealed nothing to indicate renal dise[ase]
They were kept in individual roomy cubicles and fed throughout the entire exp[eri]
mental period on purina dog chow, a complete food adequate to maintain ad[ult]
dogs in good state of nutrition.   The amount of water was not limited.

To effect a narrowing of the main renal artery a clamp was devi[sed]
whereby the degree of constriction of the vessel could be varied a[nd]
controlled.   Various contrivances were devised for this purpose b[ut]
the clamp illustrated diagrammatically in Text-fig. 1, was fina[lly]
found the most satisfactory.

The Clamp.—The entire clamp is made of one type of pure silver.[1]   The si[de]
and back (*B*) are 0.75 mm., the compressing plate (*C*) 0.75 mm. and the remova[ble]
plate (*D*) 0.5 mm. in thickness.   The double acting screw (*A*) whereby the co[m]
pressing plate is moved is made of round silver wire measuring 3 mm. in diamet[er]
The chamber of the clamp found most suitable for dogs weighing between 10 a[nd]
20 kilos measures 3 x 3 x 6 mm.

Instruments for the Application of the Clamp.—For the purpose of applying t[he]
clamp to the renal artery, which necessitates working at considerable dept[h]
retroperitoneally, special instruments were devised, which are illustrated diagra[m]
matically in Text-figs. 2, 3 and 4.   The instrument for holding the clamp while [it]
is being applied to the vessel is shown in Text-fig. 2.   The device for the inserti[on]
of the removable plate (*D*, Text-fig. 1) which helps to encase the renal vessel in t[he]

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[1] Obtained from Baker and Co., Murray and Austin Streets, Newark.

clamp is shown in Text-fig. 3. Text-fig. 4 is a screw-driver with which to screw down the compressing plate (C) and turn the retaining screw (N) which fixes the clamp in the holder or releases it after application to the vessel.

Application of the Clamp.—The clamp, without the removable plate (D), is placed with the head of the double acting screw (A) pointing downward in the lower part of the clamp holder (Text-fig. 2, position 1) and held firmly in place by means of the retaining screw (N). The portion of the renal artery dissected out for the purpose is lifted into the upturned clamp and is then encased in the clamp by the insertion of the removable plate (D) into the clamp by means of the special instrument illustrated in Text-fig. 3. The removable plate is held tightly in the jaws of this instrument while it is being inserted in the clamp. In order to be able to compress the vessel to the desired degree by means of the compressing plate (C), the clamp holder is so fashioned that pressure upon the knob (H) in the handle of the clamp holder (Text-fig. 2) releases the part that holds the clamp so that it turns over on a hinge and inverts the clamp (Text-fig. 2, positions 1, 2 and 3). This brings the head of the screw (A) of the clamp uppermost (Text-fig. 2, position 3) and makes it accessible for the purpose of screwing down the compressing plate (C). When this has been accomplished, the retaining screw (N) is turned back and the clamp, thus released, is easily pushed out of the holder and left on the vessel which it encases and constricts to the desired degree.

Effect on Blood Flow of Constriction of Main Renal Artery

Several experiments were performed to determine approximately the effect of various degrees of clamping of the main renal artery on blood flow through the kidney. A description of one of these experiments will be given. With the animal under chloralose anesthesia, a T cannula was inserted in the main renal vein which permitted the onflow of blood through the vein or the shunting of the stream and collection of blood for the purpose of measurement of rate of flow. A 3 minute period was adopted for the individual determinations. To avoid possible effects of loss of blood on blood pressure and blood flow, the blood was immediately returned to the body through a cannula in the jugular vein. The mean blood pressure in the carotid artery was determined throughout the experiment by means of a mercury manometer and recording apparatus. A clamp was applied to the renal artery, and, after determinations of flow had been made without compression of the renal artery, the clamp was tightened to various degrees and measurements of blood flow were again made. Table I illustrates the results of one of these experiments. The effects of constriction of the renal artery are obvious. In the animals with

renal ischemia that were permitted to survive, determinations of blood flow through the kidneys could not be made. Since the size of the renal arteries and the blood pressure varied in different animals, even when they were of about equal weight, the same degree of clamping in different dogs undoubtedly had different effects on blood flow. It follows that the degree of ischemia could not be standardized and the exact extent was not known. A rough estimate of slight, moderate, severe, almost complete and complete constriction was made by observation and palpation of the vessel.

Surgical Procedure for the Production of Renal Ischemia

All operations were performed under aseptic conditions, with the animal under ether anesthesia, after a hypodermic injection of morphine and atropine. A lumbar incision was made and, by retroperitoneal approach, the main renal artery was identified and dissected out clean, near its origin from the aorta, for a distance sufficient to permit the application of the clamp. No attempt was made completely to denervate the kidney, but the nerve fibers around the artery were sectioned. During the application of the clamp, care was taken in manipulating the artery to avoid at any time complete obstruction of the renal circulation. The method of application of the clamp is described above. Silk sutures were used for closure of the wound.
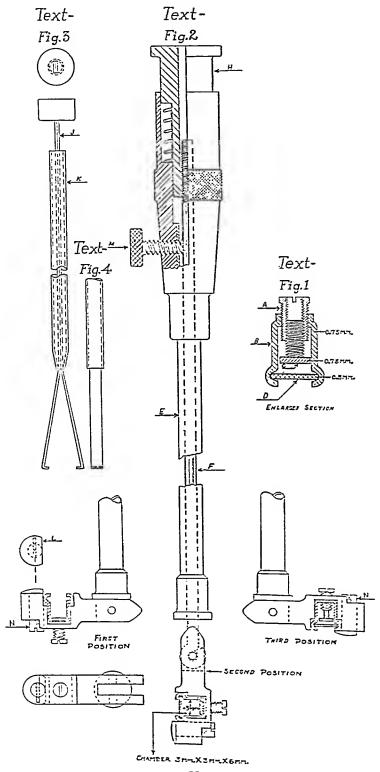
In most of the animals a clamp was first applied to the main artery of only one kidney and, after an interval of about 2 weeks, or longer, the artery of the other kidney was constricted. In most of the dogs, in the beginning, the clamps were applied so as to produce only moderate constriction of the vessels of both kidneys and subsequently they were tightened one or more times to effect a greater degree of stenosis.

TEXT-FIG. 1. The clamp. *A*, screw which carries the movable plate; *C*, movable plate; *D*, removable plate.

TEXT-FIG. 2. The clamp holder with clamp inserted. *M*, a screw, the loosening of which permits the knob *H* to be pressed down and makes it possible for the part that holds the clamp to assume any one of the three positions illustrated.

TEXT-FIG. 3. Holder for removable plate *D*, Text-fig. 1. The metal tube *K* slides down or up and tightens or loosens the grip of the metal jaws on the removable plate.

TEXT-FIG. 4. Screw-driver for tightening of screw *A*, Text-fig. 1, which carries the movable plate *C*, Text-fig. 1.

Text-Fig.3

Text-Fig.2

H

J

K

Text-M

Fig.4

Text-Fig.1

A

B

0.75mm.

0.75mm.

0.5mm.

D

ENLARGED SECTION

E

F

L

N

FIRST POSITION

THIRD POSITION

N

SECOND POSITION

CHAMBER 3mm.X3mm.X6mm.

Method of Determination of Blood Pressure

In all of the early investigations (1–6) the method of determining blood pressure is open to criticism.   This does not apply to most of the more recent studies.   In this investigation only the systolic blood pressure in a carotid artery was determined.   Carotid loops (left side) were prepared according to the method of Van Leersum (18)

TABLE I

Effect of Various Degrees of Constriction of the Main Renal Artery on the Outflow of Blood from the Renal Vein

Degree of constriction of renal artery			
None	Moderate	Severe	Very severe
Outflow of blood from renal vein in 10 sec.			
cc.	cc.	cc.	cc.
13.0	8.5	4.5	1.3
14.5	7.5	4.8	1.0
12.5	8.0	3.6	1.2
13.0	8.0	4.0	1.0
Average outflow per sec.			
1.3	0.8	0.4	0.1

This illustrates the effect of various degrees of constriction of the main renal artery on the outflow of blood from the renal vein and gives some estimate of the corresponding degrees of renal ischemia.

Dog 7.   Female.   Weight 18 kilos.   Anesthesia, ether induction followed by chloralose 0.1 gm. per kilo of body weight.   Blood heparinized *in vivo.*   Clamp on main renal artery.   Cannula in renal vein.   Moderate, severe and very severe constriction correspond to 2, 2½ and 3 complete turns respectively of screw A in the clamp.   (See Text-fig. 1.)

which permitted the daily determination of systolic blood pressure painlessly and with the animal at rest in a specially designed box. Readings of blood pressure were recorded after the animal had been resting in the box for at least 3 minutes. After this, although the pressure varied, it was not falling steadily, as it usually did during the first minute or two after the animal was placed in the box.   Instead of arbitrarily choosing the lowest of a number of determinations as the

representative pressure of the day, a questionable procedure recently suggested (19), we used the method of Dominguez (7) and recorded ten observations daily, at one sitting, all made after the period of rest. The arithmetic mean of these ten readings was considered the resting systolic blood pressure of the day. Pressures were measured at about the same time every day. The blood pressure was determined daily for a control period of at least 2 months before the first operation to produce renal ischemia. In this study all of the blood pressure determinations were made by two observers with whom the animals were familiar. In the case of Nos. 5-8, 5-9, 6-0, 6-1, 8-7 and 8-9 all of the determinations were made by one person. In Animals 2-5, 3-8, 4-9, 5-5 and 5-6 all of the determinations during the control period and for a period of several months after the initial establishment of bilateral renal ischemia and consequent elevation of blood pressure were also made by one person. In this group, during the remainder of the experimental period, the determinations were made by the person who made all of the observations in the first group mentioned above.

Determination of Renal Function before and after the Production of Renal Ischemia
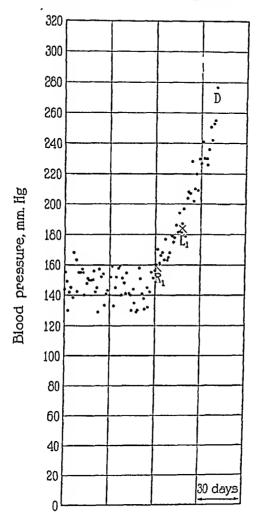
By methods described in detail in a previous study (20) urea clearance and output of phenolsulfonephthalein were determined in all of the animals at frequent intervals before and after the operations. In some of the dogs, in addition to urea, the quantity of total nonprotein nitrogen, creatinine and guanidine in the blood was also determined by standard methods.

EFFECT OF RENAL ISCHEMIA ON SYSTOLIC BLOOD PRESSURE AND RENAL FUNCTION

Text-figs. 5 to 15 illustrate the individual mean daily systolic blood pressures of all the animals and the urea clearance of some of the animals throughout the entire experimental period. Table II gives the mean systolic blood pressure in monthly periods and Table III gives the mean pressure in the entire control period and in the entire period after the initial constriction of both renal vessels.

Almost Complete Constriction of Both Renal Arteries with a Short Interval between Operations

In Animal 2-5 (Text-fig. 5 and Table II) the left renal artery was almost completely occluded at the first operation. Systolic blood
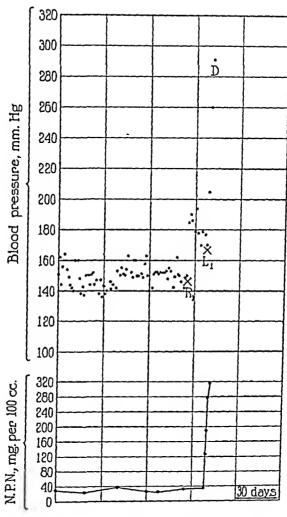
Text-Fig. 5. Dog 2-5. Initial weight 12.6 kilos. Final weight 11.2 kilos. R_1, almost complete constriction of main right renal artery; L_1, almost complete constriction of main left renal artery; D, died in uremia.

pressure soon rose and remained elevated for 16 days. Then the right renal artery was almost completely obstructed. Text-fig. 5 is a record of the systolic blood pressure before and after the operations. The

TABLE II

Mean Daily Systolic Blood Pressures during the Entire Experimental Periods

Dog No.	Months																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2-5	148	145	$R_1$174	$L_4$225	254	279	271	274	262	256	253	248	262	238	248	255	250	239
3-8	165	161	$L_4$174	$R_1$196	209	$R_2$216	208	$L_2$206	$I_3$222	211	203	$L_2$235	217	2 3	210			
4-9	157	162	$L_4$184	$R_2$217	169	173	$R_1$197	$I_4$200	$R_2$201	$L_2$219	200	190	$R_3$214					
5-5	160	151	154	$R_1$188	204	$L_4$208	200	$I_4$207	$R_2$214	202	194	$L_2$214	$N(L)_2$228	212	204	208		
5-6	152	157	154	$R_1$187	200	$I_4$222	219	216	212	201	205	212	231	233				
5-8	161	148	140	176	177	$R_2$232	$L_4$243	226	240	227	$R_2$241	$L_2$228	231	226	215	215	221	
5-9	172	157	164	176	196	$S_3$190	196	F 198	$R_1$239	$L_4$251	221	213	$I_2$231					
6-0	181	180	175	190	E 198	192	$R_1$199	$L_4$228	$R_2$235	222	214	209						
6-1	188	185	195	S 192	$L_2$262													
8-7	154	152	155	$R_1$181														
8-9	177	169	166	$A_2$166	$A_2$163	$R_3$200	190	$I_4$213	226	212								

The experimental periods are divided into intervals of 1 month. In a few instances, as can be seen in Text-figs. 5 to 15, the period is less than 1 month and usually represents either a shorter interval between operations, or one at the end of the experiment. All of the symbols have the same significance as in Text-figs. 5 to 15. Their position indicates that the procedure which they represent was carried out at the beginning of the period in which they are found.

blood pressure continued to rise following the second operation and finally reached a level almost double the mean pressure during the control period.

TEXT-FIG. 6. Dog 8-7. Initial weight 14.0 kilos. Final weight 14.2 kilos. R₁, almost complete constriction of right main renal artery; L₁, almost complete constriction of left main renal artery; D, died in uremia.

Quite suddenly, 2 days before death, the animal developed convulsions. Urea clearance, previously not materially affected, was diminished almost to zero. Blood urea and non-protein nitrogen were greatly elevated. There was a large amount of albumin in the urine. The animal became comatose and died in obvious uremia.

In the gross, the kidneys showed a moderate degree of cloudy swelling and there were two small conical areas of hemorrhagic necrosis in the cortex of the right kidney. Microscopically, in both kidneys, there was severe diffuse parenchymatous degeneration, most severe in the convoluted tubules. The infarcts of the right kidney proved to be very recent. Thrombi in small branches of the renal artery were probably present but were not demonstrated in the sections made. There were no other pathological manifestations of significance in the body.

In Animal 8-7 (Text-fig. 6 and Table II) both renal arteries were almost completely constricted, with an interval of 10 days between the two operations. After almost complete constriction of the right renal artery, the blood pressure rose and remained moderately elevated for a few days but tended then to return toward the original level. However, after almost complete constriction of the other (left) renal artery, the blood pressure soon rose again and kept increasing daily until death which occurred on the 4th day following the constriction of the second renal artery.

Urea clearance was greatly decreased after the clamping of the second renal artery and the quantities of urea nitrogen, total non-protein nitrogen, creatinine and guanidine in the blood kept increasing daily until death. There was a large amount of albumin in the urine. The animal became comatose 2 days before death, had frequent convulsions and died in obvious uremia. The kidneys were similar to those of Dog 2-5 except for the absence of infarcts.

Severe Constriction of Both Renal Arteries with a Short Interval Between Operations

In Animal 3-8 (Text-fig. 7 and Table II) the main renal artery of the left kidney was first constricted, but the clamping, though severe, was not made as tight as in Dogs 2-5 and 8-7, reported above. For 14 days following this operation there was a barely significant rise of blood pressure. Then the right main renal artery was constricted to about the same degree as the left. Following this, the pressure kept gradually increasing for over a month until it reached a high level. With considerable variations it remained at this level for about 11 months. During the next 4 months it tended toward a lower level. The animal is still alive 15 months after the constriction of both renal arteries and the daily mean systolic blood pressure is still at least 60 mm. Hg higher than the mean pressure during the control period.
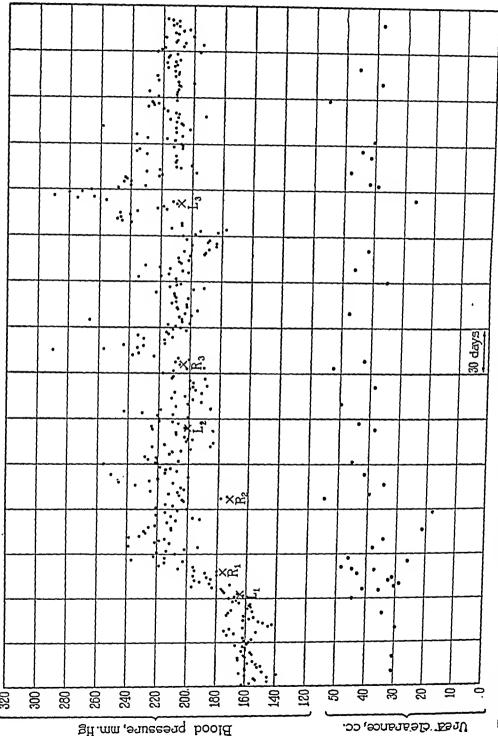
TEXT-FIG. 7. Dog 3-8. Initial weight 11.2 kilos. Final weight 7.6 kilos. L₁, severe constriction of left main renal artery; R₁, severe constriction of right main renal artery. The animal is still alive.

After the clamping of the second renal artery, urea clearance promptly became reduced to about 50 per cent of the average value during the control period (Text-fig. 7 and Table II) and, with variations, has remained at this reduced level. However, the output of phenolsulfonephthalein was not significantly affected. Throughout the experimental period the amount of creatinine, total non-protein nitrogen and guanidine in the blood remained within the limits of the control period.
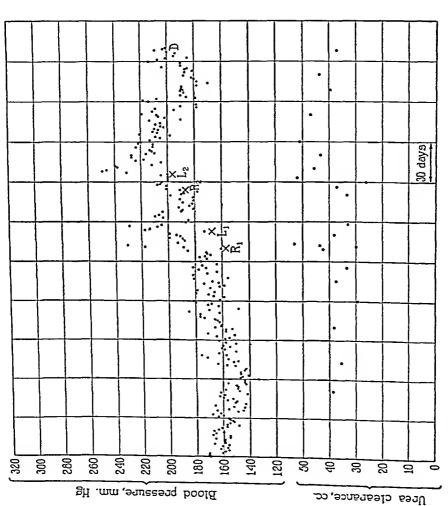
Moderate Constriction of Both Renal Arteries, Subsequently Increased

In Animals 4-9, 5-5, 5-6, 5-9 and 6-1 (Text-figs. 8 to 12, and Table II) the initial constriction of one renal artery was moderate. After an interval which varied from 11 days to 7 weeks the other main renal artery was constricted to about the same degree. In Animal 6-1 (see S, Text-fig. 12) at the first operation, clamps were applied to both main renal arteries, but the vessels were not constricted. Later the animal was again given anesthesia for a period of 2 hours (see E, Text-fig. 12). There was no elevation of blood pressure following either of these procedures. In this animal, and in the others in this group, following the constriction of one renal artery, there was a significant but variable rise of systolic blood pressure which tended to return to a lower, if not to the original level. Following the constriction of the artery of the other kidney, there occurred further increase of systolic blood pressure which remained elevated as compared with the normal but in some, after varying intervals, tended to return to a lower though not to the original level. Subsequently, after varying intervals, whether or not there was a tendency for the elevated pressure to become lower, both clamps were tightened one or more times. In one animal, No. 5-6, the stenosis of both vessels was finally made complete. (Text-fig. 10 and Table II). In all of these animals the daily blood pressure remained elevated above not only the mean pressure of the entire control period but also well above the highest values obtained during this period. These animals have been observed for periods varying from 5 months to 1 year, following the initial production of bilateral renal ischemia.
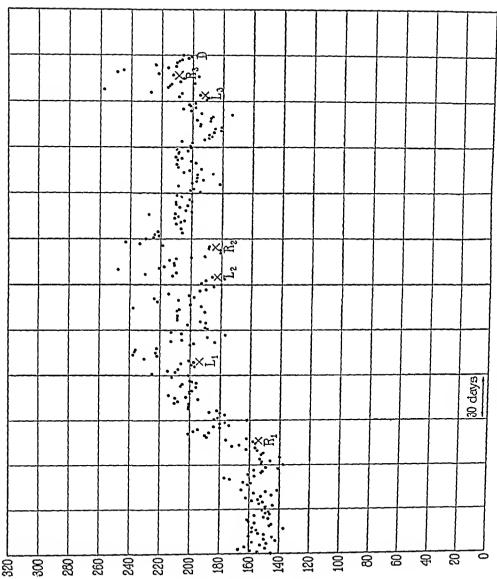
In No. 5-6, urea clearance and output of phenolsulfonephthalein were not studied because collection of urine was very difficult. In the other animals urea clearance varied greatly following the clamping of the vessels, but there was no permanently significant change and, at the end of the experimental period, the level

TEXT-FIG. 8. Dog 4-9. Initial weight 16 kilos. Final weight 17.6 kilos. L₁ and R₁, moderate constriction of left and right main renal arteries; L₂ and R₂, constriction of left and right main renal arteries increased to severe; L₃ and R₃, constriction of right and left main renal arteries again increased to almost complete. The animal is dead. It was used for the experiment to test the effect of compression of the carotid loop on systemic blood pressure.
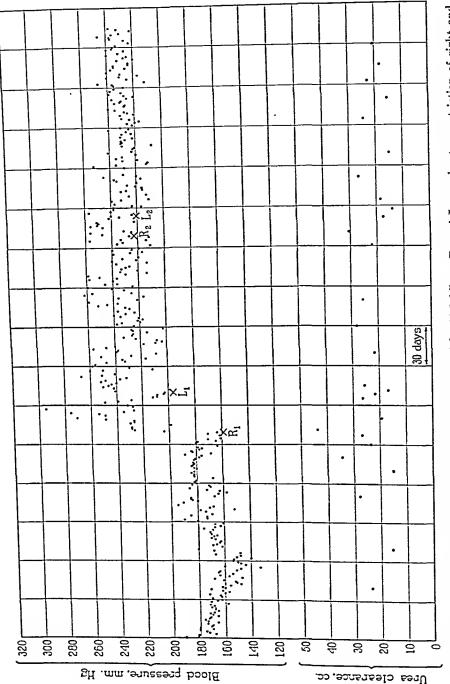
Text-Fig. 9. Dog 9. Initial weight 14.4 kilos. Final weight 16.4 kilos. R_1 and L_1, moderate constriction of right and left main renal arteries; R_2 and L_2, constriction of right and left main renal arteries increased to very severe; D, the animal died during anesthesia for proposed unilateral nephrectomy.
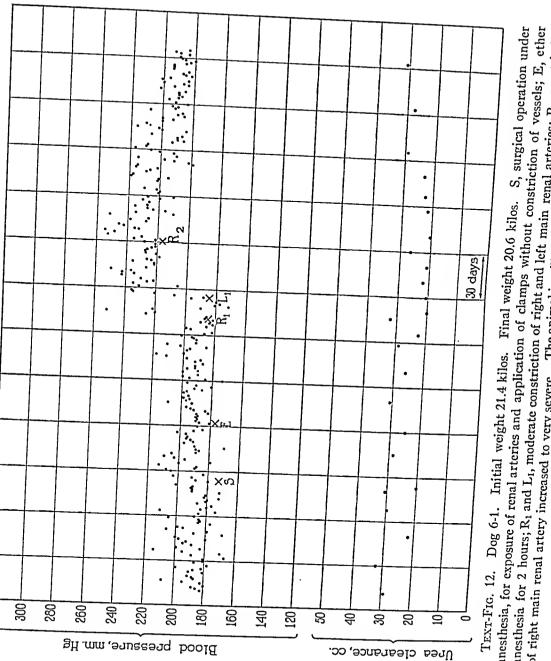
TEXT-FIG. 10. Dog 5-6. Initial weight 12 kilos. Final weight 11.6 kilos. R₁ and L₁, moderate constriction of main right and left renal arteries; L₂ and R₂, constriction of both arteries increased to severe; L₃ and R₃, constriction of both arteries made complete; D, the animal died during anesthesia for proposed unilateral nephrectomy.

TEXT-FIG. 11. Dog 5-9. Initial weight 13.2 kilos. Final weight 12.4 kilos. R_1 and L_1, moderate constriction of right and left main renal arteries; R_2 and L_2, constriction of right and left main renal arteries increased to very severe. The animal is still alive.

TEXT-FIG. 12. Dog 6-1. Initial weight 21.4 kilos. Final weight 20.6 kilos. S, surgical operation under anesthesia, for exposure of renal arteries and application of clamps without constriction of vessels; E, ether anesthesia for 2 hours; R_1 and L_1, moderate constriction of right and left main renal arteries; R_2 constriction of right main renal artery increased to very severe. The animal is still alive.

was within the limits of the control values or only slightly reduced. The quantities of urea, creatinine, total non-protein nitrogen and guanidine in the blood varied but were within normal limits throughout the entire experimental period. The output of phenolsulfonephthalein was not significantly affected in any of these dogs following the production of renal ischemia.
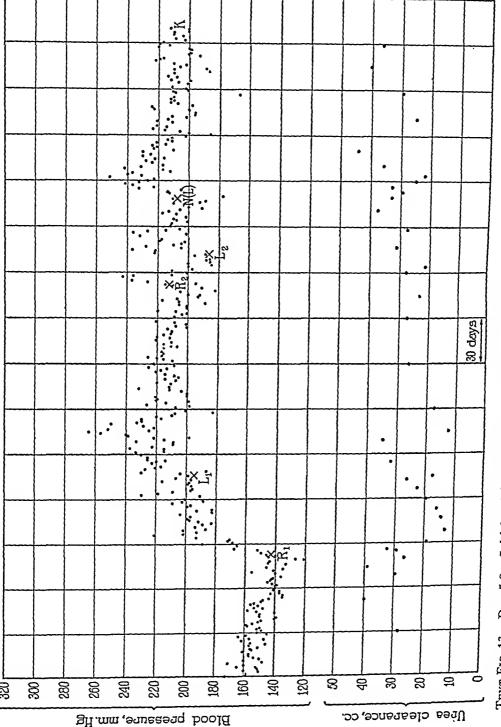
Animals 4-9, 5-5 and 5-6 are dead. Animal 4-9 was sacrificed in the experiment described at the end of this paper for the purpose of determining the effect of compression of the carotid loop on systemic blood pressure. At autopsy, the left kidney weighed 39 gm. and the right 33 gm. The capsule was moderately thickened and quite adherent in both. The microscopic examination has not yet been made.

Animal 5-5 died during anesthesia for proposed unilateral nephrectomy. At autopsy, the kidneys were about the same size as they were when observed at the preceding operations. The right weighed 44 gm. and the left 40 gm. Microscopically, both kidneys showed only moderate parenchymatous degeneration and slight thickening of Bowman's capsule and of the basement membrane of some of the glomeruli. There were more and larger vessels in the renal capsule than is usual in a dog's kidney, which probably served as accessory circulation.

Animal 5-6 also died during anesthesia for proposed unilateral nephrectomy. At autopsy, both kidneys were reduced in size. The left weighed only 15 gm. and its size was about one-fourth of what it was when observed at the first operation for the production of ischemia 7 months before death. The right kidney weighed 32 gm. In both kidneys the capsule was thickened. Microscopically, there were many large vessels in the capsule, at least some of which entered the renal substance, and evidently must have acted as a substantial accessory circulation. In both kidneys, but more in the left, there was severe diffuse parenchymatous degeneration which affected most severely the medullary parenchyma, and also diffuse increase of connective tissue. In most of the glomeruli of the left and in many of the right kidney there were greatly thickened basement membranes of the glomerular tufts and thickened Bowman's capsules. In the left kidney many of the interlobular and afferent vessels had thickened walls and the size of the lumen was greatly reduced. In some of the arterioles there was hyaline degeneration of part or of all of the thickened wall. Interstitial fibrosis was greater in the left kidney.

Bilateral Moderate Renal Ischemia, Later Increased, and, Still Later, Unilateral Nephrectomy

In Animal 5-8 (Text-fig. 13 and Table II) the original constriction of both main renal arteries and the subsequent tightening of the clamps were of about the same degree as in the preceding group of dogs. Systolic blood pressure showed a significant rise, which persisted, but with a tendency to return to a lower though not the original level.

TEXT-FIG. 13. Dog 5-8. Initial weight 12.6 kilos. Final weight 10.3 kilos. R_1 and L_1, moderate constriction of right and left main renal arteries; R_2 and L_2, constriction of right and left main renal arteries increased to very severe; N (L), left nephrectomy; K, animal killed.

6 weeks after the last tightening of the clamps the left kidney was removed. This was followed by a significant but slight further increase of blood pressure which did not persist at that level. However, the blood pressure continued to be definitely elevated as compared with the control period.
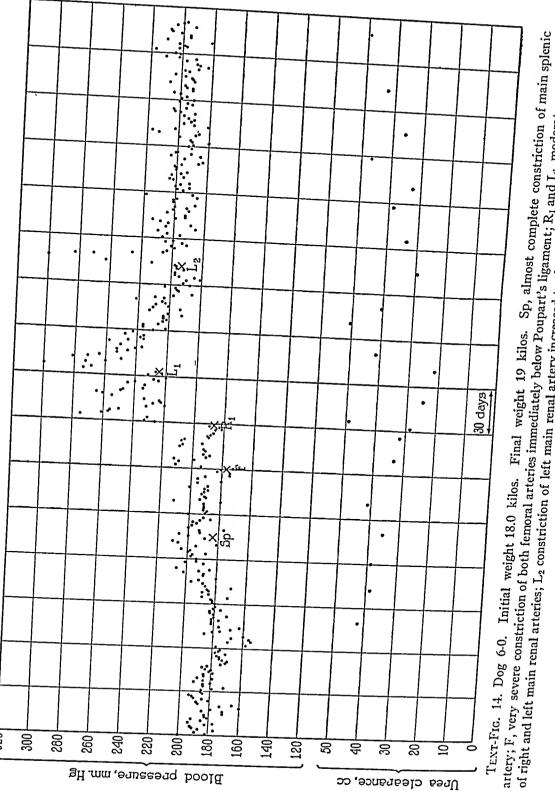
Urea clearance was moderately reduced following the initial clamping of the right renal artery but soon returned to normal. It was not significantly affected by any of the other procedures and, at the end of the experimental period, the values for the clearance and for urea, creatinine, total non-protein nitrogen and guanidine in the blood were well within the limits of the control period. The output of phenolsulfonephthalein was not significantly affected by any of the procedures. Following the tightening of the clamp a moderate degree of albuminuria developed. This became more severe after removal of the left kidney and persisted to the end. Animal 5-8 was killed 4 months after the removal of the left kidney and 10 months after the initial production of bilateral renal ischemia.

The left kidney, removed surgically, and the right, removed at autopsy, showed gross and microscopic changes similar in most respects to those of Dog 5-6. In the gross, the kidneys were only moderately reduced in size compared with their appearance at the first operation. The right weighed 30 gm. and the left 35 gm. The capsule was moderately thickened. Microscopically, the thickening of Bowman's capsules was particularly striking but, as in No. 5-6, there was also thickening of the basement membrane of glomerular tufts and of the walls of the smaller arteries. There was much less increase of interstitial connective tissue than in the left kidney of No. 5-6.

The Effect on Blood Pressure of Severe Ischemia in Other Organs, Followed by Renal Ischemia

In Animal 6-0 (Text-fig. 14 and Table II) after the usual control period, the splenic artery was almost completely occluded. 6 weeks later, both femoral arteries were very greatly constricted immediately below Poupart's ligament. There was no significant alteration of systolic blood pressure due to any of these procedures. 1 month later, the main right renal artery was moderately constricted, and 1 month after this the left renal artery was similarly constricted. About 10 weeks later the clamp on the left renal artery was tightened so that the constriction of the vessel was almost complete. A significant but moderate elevation of blood pressure occurred following the initial constriction of both main renal arteries. There was a tendency for the pressure to return to a lower level. It was temporarily in-

TEXT-FIG. 14. Dog 6-0. Initial weight 18.0 kilos. Final weight 19 kilos. Sp, almost complete constriction of main splenic artery; F, very severe constriction of both femoral arteries immediately below Poupart's ligament; R₁ and L₁, moderate constriction of right and left main renal arteries; L₂ constriction of left main renal artery increased to almost complete. The animal is still alive.

creased by the subsequent tightening of the clamp on the left renal artery. Although it soon returned to a lower level, yet this continued to be significantly but only slightly higher than during the control period. Fig. 1 is a roentgenogram of Animal 6-0, taken during life, with all of the clamps applied.

Urea clearance, the quantity of urea, creatinine, total non-protein nitrogen and guanidine in the blood, and the output of phenolsulfonephthalein were not significantly affected by any of these procedures. At the end of the experimental period their values were about the same as during the control period.
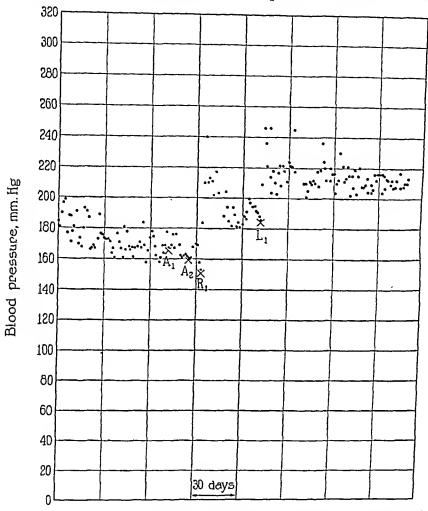
Renal Ischemia Following Excision of Right Suprarenal Body, Section of Left Splanchnic Nerves, Denervation of the Left Suprarenal Body and Destruction of Its Medulla

In Animal 8-9 (Text-fig. 15 and Table II) after the usual control period, the right suprarenal body was removed. Under ether 2 weeks later the left major and minor splanchnic nerves were cut, all of the nerve fibers to the left suprarenal body that were recognizable in the gross were severed, and the medulla of the left suprarenal body was removed as completely as possible by means of a burr. The blood pressure remained practically unchanged after these procedures. Then, at separate times, both main renal arteries were moderately constricted by clamps. Following this the systolic blood pressure showed a moderate rise which persisted with some tendency to return to a lower but not to the normal level for this dog. In No. 8-9, for the same reason as in No. 5-6, urea clearance and output of phenolsulfonephthalein were not determined. The quantity of urea, creatinine, total non-protein nitrogen and guanidine in the blood varied but was not significantly affected by any of the procedures.

The Possible Effect of Compression of the Carotid Loop on Systemic Blood Pressure

An experiment was performed which was designed to cover the possibility that the elevated systolic blood pressure found in all of the animals during the period following the constriction of both renal arteries was really due in some way to compression of the carotid loop by the cuff used in making the determinations. Although the same method was used during the control period, yet it might be averred

that the clamping of the renal arteries altered the body in some way so as to account for this difference of reaction to a possible reflex from the compressed carotid artery, or to the partial reduction of cerebral

TEXT-FIG. 15. Dog 8-9. Initial weight 13.6 kilos. Final weight 16.2 kilos. A_1, excision of right suprarenal body; A_2, destruction of medulla and denervation of left suprarenal body. Section of left major and minor splanchnic nerves; R_1 and L_1, moderate constriction of the right and left main renal arteries. The animal is still alive.

circulation caused by the repeated, momentary occlusion of the one carotid artery during the determination of blood pressure.

Animal 4-9 was used. At this time this animal was still showing a significant though slight elevation of blood pressure as determined by the carotid loop method.

Under ether anesthesia, after a hypodermic injection of 1/2 grain morphine sulfate and 1/300 grain atropine sulfate, a cannula was inserted in the right femoral artery and connected with a mercury manometer for the purpose of making a kymographic record of the mean blood pressure in this vessel. Through a small incision in the neck, the right common carotid artery was exposed and separated from surrounding tissues. Some determinations of pressure in the carotid loop (left common carotid) were made and the cuff was then left loosely applied to be in

TABLE III

Mean Daily Systolic Blood Pressure during the Control Period before Clamping Either Renal Artery and during the Entire Period after the Initial Constriction of Both Main Renal Arteries

Dog No.	Control period			Period after initial constriction of both renal arteries		
	Mean systolic blood pressure	No. of days	No. of observations	Mean systolic blood pressure	No. of days	No. of observations
	mm. Hg			*mm. Hg*		
2-5	146	62	62	174	21	21
3-8	160	63	63	252	454	345
4-9	159	63	63	215	363	314
5-5	161	161	145	202	138	116
5-6	154	76	76	205	201	172
5-8	150	83	83	214	294	219
5-9	169	148	148	232	274	235
6-0	185	134	121	224	227	194
6-1	191	182	156	221	161	137
8-7	152	86	68	262	4	4
8-9	173	72	65	212	98	87

By number of observations is meant the number of days on which determinations of blood pressure were made. During the control period, in many of the dogs pressures were taken every day of the week, so that the number of days and number of observations coincide. The mean daily systolic blood pressure is the mean of ten observations made at one sitting.

readiness for the compression of the loop. A record of blood pressure in the femoral artery was then made. The mean pressure in the femoral artery immediately before the compression of the carotid loop was 185 mm. of mercury and 205 mm. in the carotid loop. This is about the normal difference between the pressures in these two vessels when both are obtained by direct methods. The carotid loop was then quickly compressed, by pumping air into the cuff around it, so as completely to obstruct the flow of blood through the left carotid artery for 80 seconds. During the compression of the carotid loop (see 1, Fig. 2) the mean

pressure in the right femoral artery showed an insignificant elevation of not more than 5 mm. Hg. After an interval, the freshly exposed right common carotid artery was completely obstructed by compression with a bulldog clamp, the jaws of which were covered with rubber, and, at the same time, the carotid loop (left common carotid) was compressed so as completely to obstruct blood flow through the vessel. There was a prompt but slight (15 mm. Hg) rise of pressure in the femoral artery, which persisted while the carotids were obstructed but promptly returned to the original level when the compression of both vessels was released (see 2, Fig. 2). After an interval of a few minutes the left carotid alone was again completely obstructed by compression of the carotid loop. This time there was practically no effect on the mean blood pressure in the femoral artery (see 3, Fig. 2). The compression of the carotid loop was released and, after an interval, the freshly exposed right carotid alone was clamped. There was a prompt but slight (15 mm. Hg) elevation of mean blood pressure in the femoral artery (see 4, Fig. 2) which persisted until the clamp was removed from the artery, when it promptly returned to the original level. The interesting finding is that the increase of pressure during the obstruction of this freshly exposed right carotid artery alone was exactly equal to the elevation which occurred following the previous compression of both carotid arteries. It follows, therefore, that the compression of the carotid loop contributed little or no effect when both vessels were compressed at the same time.

All of these observations were repeated with exactly the same results (see 5-9, Fig. 2). Even ten quickly repeated occlusions of the left carotid artery in the carotid loop (see 6, Fig. 2) failed to have a significant effect on the pressure in the femoral artery. Constriction of the freshly exposed right carotid artery with or without coincident compression of the carotid loop again had an equal effect on the blood pressure in the femoral artery.

It is safe to conclude, therefore, that the elevation of blood pressure (of the order found following the constriction of the renal arteries Text-figs. 5 to 15) cannot be attributed to any effects of compression of the carotid loop in making the determination.

DISCUSSION

The results of the foregoing experiments show that the constriction of the main arteries of both kidneys of dogs was followed invariably by elevation of systolic blood pressure, determined in a carotid artery, which persisted for as long as 15 months. Great, permanent constriction of large arteries in other parts of the body did not induce persistent significant elevation of systolic blood pressure. The in-

:reased values cannot be accounted for by the compression of the :arotid loop in making the determinations.

The mechanism of elevation of the blood pressure of these animals 1as not been elucidated by these experiments. Bell and Pedersen (10), in attempting to explain the temporary increase of systolic blood pressure which they observed in rabbits, following the experimental production of venous stasis in one kidney, used the teleological argument that: "When there is increased resistance in the renal circulation the blood pressure must be increased in order to maintain the normal blood flow." They referred to this as a compensatory process. Such an argument could also be applied to the case of experimental renal ischemia and has the advantage as well as weakness of all teleological reasoning. It fails to explain just why the normal blood flow must be maintained and gives no clue to the specific mechanism whereby this is effected. It merely takes advantage of the fact that in those animals that survived the blood pressure actually was raised and, *as a consequence*, blood flow was most probably improved; but it does not explain the mode of development of the elevation of blood pressure.

It is a simple matter to outline a number of possible mechanisms whereby the blood pressure may be raised as a result of renal ischemia.

(*a*) Afferent impulses from the affected nerve endings in the ischemic kidneys to the sympathetic ganglia or vasomotor center may result in general vasoconstriction and consequent elevation of blood pressure.

Complete denervation, if possible, of both kidneys, before the application of the clamps, would give some clue to the existence of such impulses but would not distinguish between a direct, purely nervous, and an indirect humoral mechanism brought into play by such afferent impulses from the kidney. This experiment has not yet been performed.

(*b*) Afferent impulses from the ischemic kidneys may, in some way, bring about increased output of some internal secretion which, by peripheral or central action, may effect general vasoconstriction, and thus raise the blood pressure.

Sufficient experiments to determine this were not made. That the suprarenal medulla probably plays no part in the process is indicated by the experiment on Dog 8-9 in which the right suprarenal body was removed, the medulla of the left destroyed and the left splanchnic

nerves and nerve fibers to the left suprarenal body sectioned. The blood pressure was not significantly affected by these procedures. Subsequently, production of moderately severe bilateral renal ischemia was followed by moderate elevation of systolic blood pressure. The possible part played by the pituitary body was not investigated.

(c) There may be an accumulation or new formation of some substance, or there may occur a disturbance of chemical equilibrium between substances present in the blood which may effect a pressor action like that of a hormone.

The existence in the blood of subjects with hypertension of a substance or substances capable of raising the blood pressure and differing either quantitatively or qualitatively from any found in subjects with normal blood pressure, has not been demonstrated. In those animals (Nos. 2-5 and 8-7) in which the constriction of the vessels was almost complete from the beginning, that showed great elevation of blood pressure but did not survive very long, there was an accumulation of urea, creatinine and total non-protein nitrogen in the blood. However, it has not been shown that these substances have a pressor effect and they did not increase in the blood of the animals in which the clamping of the renal arteries was less severe but in which, nevertheless, an elevation of pressure did occur. In all of the animals, except No. 2-5, the quantity of guanidine in the blood was determined at intervals. Animal 8-7 is the only one that showed an appreciable elevation of this substance above the normal. This occurred during the 4 days of survival following the almost complete clamping of the second renal artery, during which time the animal was in uremia. In all of the other animals, even in No. 3-8, which had the longest period of persistent hypertension, during which pressures even higher than those of Animal 8-7 were attained, no increase of guanidine in the blood was found. Major (21) has shown that there is decreased excretion of guanidine in the urine of patients with arterial hypertension. This was not studied in these animals. Major and collaborators (22–27) have shown that various guanidine compounds, when injected into the blood stream of animals, cause a rise of blood pressure which persists longer than after injections of adrenalin. It has been shown (28) that the site and mode of action of guanidine compounds are like those of adrenalin. However, no one has found an increase of guanidine in

the blood of patients with vascular hypertension. The exact part, if any, played by the metabolism of guanidine in patients with hypertension, and in these animals with experimentally elevated blood pressure, still remains to be determined.

RÉSUMÉ

In eleven dogs, by the carotid loop method of Van Leersum, systolic blood pressure was determined daily for at least 2 months before, and for from 3 days to 15 months after, the constriction of both renal arteries by means of a special clamp devised for the purpose. The clamp permitted the degree of constriction of the vessel to be varied and increased at will. In some of the animals the constriction was made great from the beginning; in others, it was made moderate at first and subsequently increased one or more times. Constriction of one renal artery was followed by a moderate or slight rise of blood pressure which tended to return toward the level of the control period. Following the production of bilateral renal ischemia, the systolic blood pressure rose to a varying degree in all of the animals. During a variable period following the constriction of the second vessel very high values were common. No attempt was made in this series of animals to reduce or remove the accessory circulation through the capsule of the kidney. This is being done in another series of animals. In those animals in which the constriction was made moderate at first, and later increased, the amount of this accessory circulation probably became quite considerable and very likely was responsible for the tendency of the elevated pressure eventually to decrease in most of the animals. However, in all of the animals, the systolic blood pressure remained elevated well above the normal range.

In two animals, (Nos. 2-5 and 8-7), in which the clamping of both arteries was made almost complete from the start, the rise of blood pressure which followed was accompanied by the development of uremia which rapidly proved fatal. In these animals, the amount of urea nitrogen, total non-protein nitrogen and creatinine in the blood kept increasing and the urea clearance and output of phenolsulfonephthalein kept decreasing until death. In the remaining animals that survived for many months the only renal function test which indicated some renal damage in a few of the animals was the urea clearance.

Urea, total non-protein nitrogen, creatinine and guanidine remained within normal limits. In one animal (No. 3-8), that has had a persistently elevated systolic blood pressure for 15 months following severe constriction of both main renal arteries, the urea clearance remained reduced throughout to about 50 per cent of the mean value obtained during the control period. In the remaining animals in which the constriction was made moderate at first and then increased, urea clearance showed either slight reduction, with rapid return to normal, or practically no change from the normal.

In one animal (No. 6-0), the splenic artery and both femoral arteries were greatly constricted at different times before the clamps were applied to the renal arteries, but no rise of blood pressure occurred until after the renal arteries of this animal had been constricted.

In one animal (No. 8-9), the right suprarenal body was removed, the left suprarenal body was denervated and its medulla mechanically destroyed, and the left major and minor splanchnic nerves were sectioned. The blood pressure of this animal showed no significant change until after the renal arteries were constricted, when a moderate rise promptly occurred and persisted.

Of the nine animals in which the systolic blood pressure remained elevated for 4 months or longer following the initial constriction of both renal arteries, five are still alive. The examination of the kidneys of three of the dead animals (Nos. 5-5, 5-6 and 5-8) in this group (one kidney of No. 5-8 was removed surgically) indicates that ischemia may be able to induce significant changes in glomeruli, parenchyma and vessels of the kidney. Gross infarction of kidney substance was not observed in these kidneys, and microscopically massive necrosis was not present. The changes in the tissues of the animals with persistent hypertension and without signs of uremia were therefore abiotrophic rather than necrobiotic. Thus, necrosis of kidney substance was not a necessary condition for the development of elevated blood pressure in these animals. It is to those abiotrophic changes in the kidneys that the elevation of blood pressure is probably attributable because it is well known that in acute experiments clamping even of both renal vessels has little or no immediate effect on blood pressure. The pathological changes in the kidneys as well as possible pathological changes in other parts of the body will be studied at autopsy in the remaining animals and will be the subject of another report.

Throughout this study cardiograms of all the animals were made at regular intervals as an aid to determine the development of cardiac hypertrophy in these animals. The results of the study to determine the existence of hypertrophy by roentgenographic and morphologic methods will be included in the report which will deal with the anatomical findings in all of these animals.

CONCLUSIONS

These experiments indicate that, in dogs at least, ischemia localized to the kidneys is a sufficient condition for the production of persistently elevated systolic blood pressure. When the constriction of both main renal arteries is made only moderately severe in the beginning, the elevation of systolic blood pressure is unaccompanied by signs of materially decreased renal function. In this respect the hypertension in these animals resembles the hypertension which is associated with so called benign nephrosclerosis in man. Subsequent increase of the constriction of the main renal arteries does not materially damage renal function, probably because of adequate development of accessory circulation. More delicate methods for detecting a change may yet prove that some damage does occur. Almost complete constriction of both main renal arteries, from the beginning, results in great elevation of systolic blood pressure which is accompanied by severe disturbance of renal function and uremia. This resembles the type of hypertension which is associated with so called malignant nephrosclerosis, in the sense of Fahr (17). In several of the animals with persistent elevation of systolic blood pressure, anatomical changes were observed in the glomeruli, vessels and parenchyma of the kidneys which are most probably directly referable to the ischemia.

It is hoped that these investigations will afford a means of studying the pathogenesis of hypertension that is associated with renal vascular disease.

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EXPLANATION OF PLATES

PLATE 23

FIG. 1. Dog 6-0. Roentgenogram, showing an arterial clamp on the splenic, both renal and both femoral arteries.

PLATE 24

FIG. 2. DOG 4-9. Ether anesthesia drop method, following hypodermic injection of morphine and atropine. Experiment to determine the effect of compression of the carotid loop on systemic blood pressure.

The numbers 1 to 9 indicate the beginning of obstruction of one or both carotid arteries. The release of the signal indicates when the compression was stopped.

(1) Left common carotid artery completely obstructed by compression of carotid loop by means of air pumped into a cuff around the loop.

(2) Freshly isolated right common carotid and the left common carotid in the loop simultaneously obstructed.

(3) Same procedure as (1).

(4) Freshly isolated right common carotid alone completely obstructed.

(5) Same procedure as (1).

(6) Left common carotid obstructed by compression of loop as (1) but repeated ten times. Each obstruction lasted 10 seconds, with an interval of 10 seconds between occlusions.

(7) Same procedure as (4).

(8) Same procedure as (1).

(9) Same procedure as (2).

Fig. 1

FIG. 2
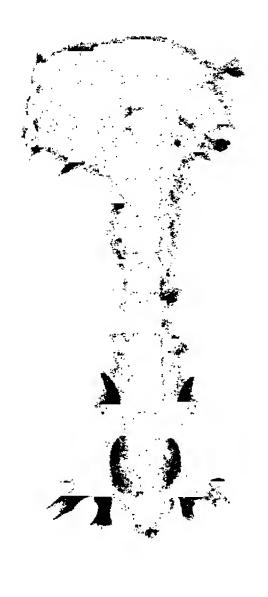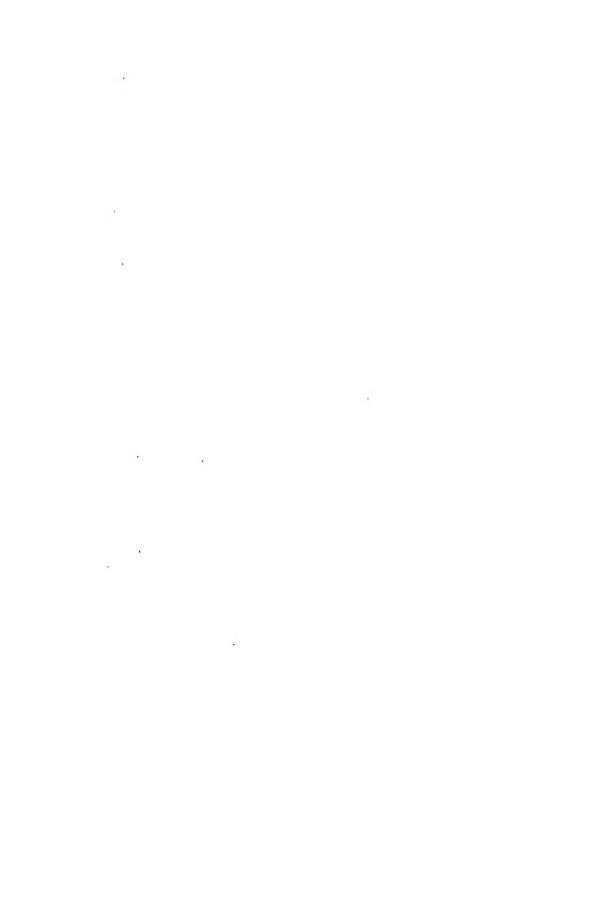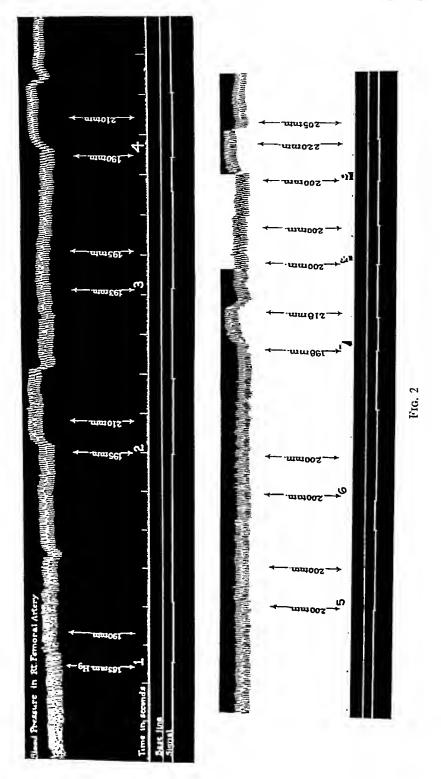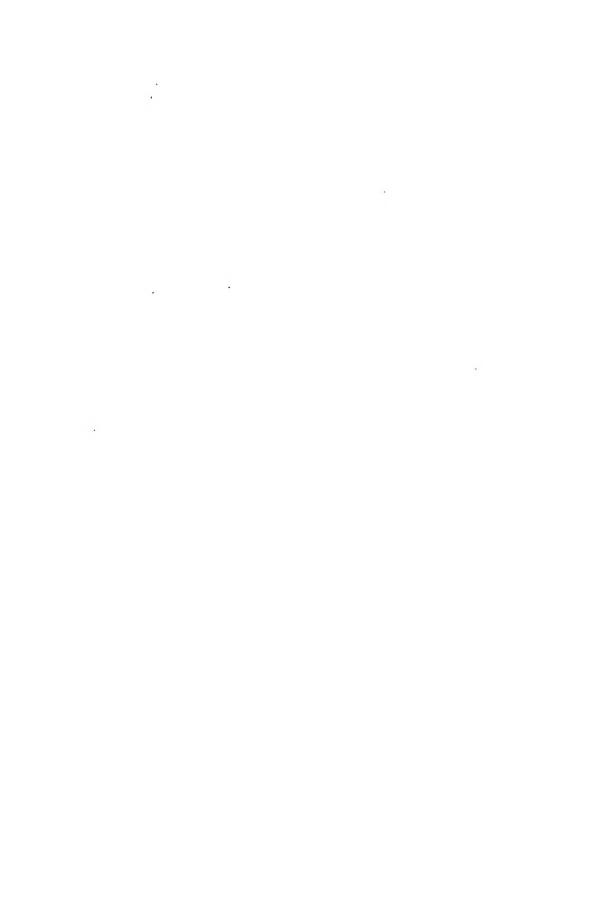
BACTERIAL GROWTH AND MULTIPLICATION AS DIS-CLOSED BY MICRO MOTION PICTURES

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PLATES 25 TO 28

With an apparatus and technique developed primarily for studying the injurious effects of ultraviolet and X-radiation on microorganisms, long series of micro motion pictures have been made during the last 2 years of many kinds of bacteria. Besides providing numerous illustrations of normal growth and multiplication, these records show the development and the subsequent behavior of many morphological structures which have been described as evidence that these organisms go through complex cycles of growth. They demonstrate the origin of much of the pleomorphism seen in bacterial cultures and they bring to light many relationships between different bacteria, and especially between bacteria and certain higher microorganisms, which otherwise are not apparent. Though the present films contain answers to only a few of the many questions of bacterial life history which in recent years have received a detailed, but mainly imaginative, treatment, it is clear that the micro motion picture study of living microcultures allows a direct experimental attack upon them. The following discussion is limited to a description of the multiplication processes occurring in bacteria and to an outline of some relationships between these processes and the pleomorphism of several common bacterial types.

In spite of the fact that it is an old technique, bacterial microculture on solid media has found little serious use. Many years ago it was employed[1] in observing the division of diphtheroids; very recently it

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[1] Hill, H. W., *J. Med. Research*, 1902, **7**, 202. Kisskalt, K., and Berend, E., *Centr. Bact. I. Abt., Orig.*, 1918, **81**, 444.

has been used in micro motion picture studies[2] of the rate of bacterial multiplication and of the germination of certain aerobic spores. The growth of single avian and human tubercle bacilli has been observed in fluid microcultures so small that the fate of individual cells could be determined[3] and a procedure, somewhat like the one used in the present study, has been applied to the development of some microcolonies[4] and to the cultivation of several anaerobic bacteria and their spores.[5] The present photographs have been made of bacteria growing on the surface of thin transparent blocks of solid media, melted on cover-glasses and inverted and sealed over hollow ground slides. These micropreparations are very easy to make and are of general applicability. They have already been described in studies of the pleomorphism of *B. shigae*,[6] of the growth of *Mycobacterium phlei*[7] and of several strains of the tubercle bacilli from cold blooded animals.[8] The micro motion picture equipment with which the development in these preparations has been followed has also been described.[9]

All of the films serving as data for the following discussion have been made with 3 mm. oil immersion objectives upon fine grained 35 mm. motion picture film, the initial magnification being about 350 ×. The interval between exposures varied between 5 seconds, for some rapidly multiplying staphylococci, and 8 minutes, for some of the mycobacteria. Including those which form the basis for the descriptions already published, these motion picture records now cover approximately 40,000 feet of negative and represent an observation period of several thousand hours.

The cultures have been drawn from a variety of sources. The *B. shigae*, *M. phlei* and tubercle bacillus strains were enumerated in the papers dealing with these organisms. Other bacteria, together with the media upon which they have been photographed, are the following: (1) *B. megatherium*. From American Type Culture Collection, Chicago (A.T.C.); on ordinary nutrient agar (N.A.). (2) *B. subtilis*. A.T.C.; N.A. (3) *B. mycoides*. A.T.C.; N.A. (4) *B. flexus* (?). A

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[2] Bayne-Jones, S., and Adolph, E. F., *J. Cell. and Comp. Physiol.*, 1932, 1, 409; 2, 329.

[3] Kahn, M., *Am. Rev. Tuberc.*, 1929, 20, 150; *Ann. Inst. Pasteur*, 1930, 44, 259.

[4] Schubert, O., *Centr. Bact., 1. Abt., Orig.*, 1920, 84, 1.

[5] Fortner, J., *Centr. Bact., 1. Abt., Orig.*, 1930, 115, 96.

[6] Wyckoff, R. W. G., *J. Exp. Med.*, 1933, 57, 165.

[7] Wyckoff, R. W. G., and Smithburn, K. C., *J. Infect. Dis.*, 1933, 53, 201.

[8] Wyckoff, R. W. G., *Am. Rev. Tuberc.*, in press.

[9] Wyckoff, R. W. G., and Lagsdin, J. B., *Rev. Scient. Instruments*, 1933, 4, 337.

laboratory spore former closely resembling *B. flexus*; N.A., N.A. + 6 per cent glycerine, Long's synthetic medium + agar (L.A.). (5) *B. pyocyaneus* (*Pseudomonas aeruginosa*). A.T.C.; N.A. (6) *Pseudomonas fluorescens*. From Dr. K. Landsteiner; N.A. (7) *B. lactis aerogenes*. A.T.C.; N.A. (8) *B. prodigiosus* (*Serratia marcescens*). A.T.C.; N.A. (9) *Klebsiella pneumoniae* (Friedländer's bacillus). From Dr. O. T. Avery; on dextrose agar (D.A.). (10) *Neisseria catarrhalis*. A.T.C.; N.A. (11) *Azotobacter beijerinckii*. A.T.C.; on mannose agar (M.A.). (12) *Azotobacter chroococcus*. From United States Department of Agriculture; M.A. (13) *Rhizobium radicicola*. From United States Department of Agriculture; M.A. (14) *Diplococcus pneumoniae*. From Dr. O. T. Avery; Types I, II and III and rough strain; on serum agar, immune serum agar and D.A. (15) *Streptococcus pyogenes*. Two strains from A.T.C., one growing in long chains, the other showing short chains in smears but growing in staphylococcoid clumps and showing many irregular diphtheroid-like divisions; N.A. and D.A. (16) *Staphylococcus aureus*. From Dr. P. K. Olitsky; N.A. (17) *Micrococcus luteus*. A.T.C.; N.A. (18) *Sarcina lutea*. A.T.C.; N.A. (19) *Rhodococcus roseus*. A.T.C.; N.A. (20) *Rhodococcus agilis*. Laboratory contaminant; N.A. (21) *Corynebacterium pseudodiphthericum* (*C. hoffmannii*). From New York State Department of Health; N.A. (22) *Corynebacterium pseudotuberculosis murium*. From New York State Department of Health; N.A. (23) A laboratory contaminant which closely resembles the non-acid-fast soil mycobacteria but is not identical with any described in D. H. Bergey, Manual of determinative bacteriology; N.A., D.A. and G.A.

Photographs have been made of several actinomyces, of non-acid-fast soil bacteria intermediate between them and the mycobacteria and of yeasts and endomyces. These pictures bring out relationships to bacteria which will be described in a later paper. It is to be emphasized that some of the pictures already made are in a sense exploratory: they show that many kinds of bacteria can profitably be studied in microculture but they probably do not describe in full the modes of growth of all the bacteria to which they have been applied.

The Multiplication of Bacteria

If the multiplication of bacteria is examined in the motion pictures, it is immediately evident that the process of division is not always the same. Some bacteria provide perfect examples of transverse fission; some multiply by what may be called coccoid division; others by a budding or sprouting. For still others the division mechanism is something intermediate between these three extremes. In fission, an organism elongates and then splits into two shorter rods; in coccoid division, a coccus swells more or less uniformly as it grows and then breaks down, usually quite suddenly, into two smaller spheres; in

budding, a portion of the cell wall apparently ruptures, a small amount of protoplasm is extruded and develops into a new cell before being pinched off. Most simple bacteria divide exclusively by fission. Examples are members of the colon-typhoid group and both aerobic and anaerobic bacilli (Figs. 1–4, 5–7). Staphylococci furnish ideal illustrations of coccoid division (Figs. 8–11, 12–15). Budding is most often observed among the diphtheroids and the mycobacteria (Figs. 21–24) which may in fact show all three modes of division. Some cocci, obviously related to the corynebacteria, divide at first by a sprouting that eventually is replaced by coccoid division (Figs. 30–33). During their period of most active growth the higher bacteria are long rods or even filaments. At this stage division is by fission or budding. In these, and many other, bacteria multiplication goes on unabated for some time after growth has slackened. As a result any filaments that may be present fractionate into rods which in turn become shorter and shorter (Figs. 25–27). Sometimes this division is prolonged until the original rods look like short chains of cocci (Figs. 28–29). Often indeed these short organisms round out into true spheres which when they resume growth on fresh medium do not at first function as rods. A few swell and divide several times by true coccoid division; more often they bud or sprout thin filaments that eventually develop into normal rods.[10]

Factors Responsible for Pleomorphism

The factors which can be seen at work in determining the natural pleomorphism of these higher bacteria are the same ones which result in an induced pleomorphism of many simpler organisms. Two, the most important, may be defined by saying (1) that bacteria can divide in more than one way and (2) that the rate of division can vary independently of the rate of growth. This growth rate, and perhaps also the rate of division, are strongly influenced by the kind and richness of the medium upon which the bacteria are living; it is therefore to be anticipated that the observed bacterial morphology will depend upon the medium used as well as upon the frequency with which the bacteria are transferred to fresh food material. Among the higher

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[10] See the pictures of *M. phlei* reproduced as Figs. 12–14, 16–18 in the article by Wyckoff and Smithburn.[7]

bacteria frequent transplants on a rich medium stimulate greatly the growth rate without correspondingly accelerating the rate of division. Young cultures thus produced commonly consist of comparatively long rods and even filaments with many branching forms. This stage of rapid growth is nearly or entirely suppressed if a poor medium is used or if transplants are made only at infrequent intervals. Under such circumstances the bacteria are all short rods which soon pass into the coccoid forms of old age.

The pleomorphism of cocci, due mainly to resemblances to certain corynebacteria, is to be ascribed to the operation of the first of the foregoing factors. Among the other simpler bacteria, shape of an uninjured organism is more dependent upon the ratio between the rate of growth and the rate of division. Some bacilli always divide at their midpoints, giving rise in this way to colonies that have organisms very uniform in size. Some strains of colon-typhoid bacteria and of spore-bearing bacilli are examples (Figs. 1–4). Most rod-forming organisms are not so regular in shape; their actively growing cultures are a heterogeneous mass of long and short rods.

Many spore formers (Figs. 34–36, 37, 40) and bacteria like *B. pyogenes* (Figs. 38–39) and *B. prodigiosus*, show a natural pleomorphism of this type. Very short rods, like *N. catarrhalis* (Figs. 41–42) and the rough pneumococcus (Fig. 55), are for the most part uniform in size but like most of these other bacteria, their old cells are smaller than those which are actively growing. The living elements in old bacterial cultures are not, however, always small cells. Thus the last viable bacteria in old broth cultures of *B. shigae* are filaments[11] which may grow to be several tenths of a millimeter long. Whatever the old age forms in a culture may be they always develop normally when planted on fresh medium.

The shapes of many bacteria can be altered merely by changing the composition of the medium upon which they are cultivated. For example the rods of *B. flexus* (?), which are short and straight on ordinary nutrient agar become gnarled and twisted (Fig. 43) if glycerine is added to the medium. It is easy to induce a pronounced pleomorphism in many members of the colon-typhoid group of organisms. Forms of the dysentery bacillus (*B. shigae*) obtained by cultivating it

[11] See Fig. 4 in the article by Wyckoff.[6]

in LiCl-containing and in other special media have already been de-
scribed and pictured. In shape they resemble those seen amongst the
higher bacteria but they occur not as youth and old age forms but as
stages in the adaptation of the bacteria to a new and unaccustomed
environment. Most of the swollen, distorted and macrococcoid cells
that are common under such conditions have the appearance of being
injured organisms; if moderately distorted they multiply with the
ultimate production of normally shaped bacilli; if too swollen they
disintegrate through rupture of their cell membranes. Similar bizarre
forms are common in old cultures of many spore-bearing aerobes
(Figs. 40, 44–45). Their motion pictures show that they are stages
in the disintegration of normal rods. Though sometimes treated as
examples of life cycle forms, their behavior in the films is that of
grossly injured bacteria or of already dead bits of protoplasm.

One of the most frequently discussed features of bacterial pleo-
morphism is branching. Among the higher bacteria rapidly growing
filaments may extrude protoplasm from several points along their
lengths. These multiple branched structures are identical in appear-
ance and general behavior with typical actinomycelial threads (Figs.
23–27). The acid-fast mycobacteria usually branch only at one
point of a rod, but the mechanism of this branching seems to differ
in no essential way from that which occurs along the filaments of the
other higher bacteria (Figs. 46–53). Branching rods are frequent in
rapidly growing smooth strains of the tubercle bacilli; they have been
occasionally observed in microcultures of their rough dissociants.
Forms that in both mode of production and subsequent behavior are
not essentially different from these tubercle bacilli occur with other
pleomorphic elements in cultures of *B. shigae*.[12]

Micro motion picture studies provide a convenient way of deciding
whether the coccobacilli are more naturally to be considered as short
rods or as true cocci. Thus *N. catarrhalis* divides by elongation and
fission and is obviously a short rod (Figs. 41–42). It is interesting
that the different types of pneumococci, which also divide by fission,
can readily be distinguished in the pictures. Type I organisms break
up into diplococci almost as soon as formed, those of Type III produce
streptococcoid chains (Fig. 56) that in young and frequently trans-

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[12] *Cf.* Figs. 8, 9, 15, 16, etc., in the article by Wyckoff.[6]

planted cultures are very long. Any tendency towards splitting into diplococci manifests itself very late. As might be expected Type II is intermediate. It is evident that these differences as well as the distinctions that exist between streptococci, staphylococci, sarcinae, etc. are determined in the main by whether the two bacteria resulting from a division are completely separated as soon as formed or whether this separation is effected only after further growth has taken place.

Factors Influencing the Morphology of Colonies

The manner of division is one of the most important factors in fixing the shape and general appearance of macroscopic bacterial colonies. The operation of this factor is clearly evident in the motion pictures. In them four limiting types of colony growth must be distinguished: (1) mucoid (or mucoid-smooth), (2) smooth (or non-mucoid-smooth), (3) rough and (4) convoluted. If an organism produces a capsule (Figs. 54, 56) or a large amount of some viscous metabolic product (Figs. 46–53, 57), its colonies will be round, smooth on the surface and of a mucoid consistency; the other colony types are due to bacteria which do not produce such jelly-like exudates. If the bacteria are cocci or if they are rods which break apart cleanly when they divide (Figs. 5–7, 41, 42), they ordinarily will develop round smooth colonies; if they are bacilli which tend to produce long filaments (Figs. 34–36, 37) or which give rise to long chains of organisms through incomplete separation after division, the resulting rough colonies will reach out irregularly over the surface of the medium. If bacteria form tangled masses as they grow with no tendency to spread over the medium their colonies will be convoluted (Fig. 62). These heaped-up colonies which are typical of the "rough" strains of the mycobacteria are irregular in outline like those which are truly rough but unlike them have no thin spreading edge. Rough colonies of many shapes result from the growth of such bacilli as *B. subtilis, B. megatherium* and *B. anthracis* and from rough strains of colon-typhoid bacteria. The colonies of "rough" pneumococci, of *N. catarrhalis*, of smooth *B. shigae* and of *B. lactis aerogenes* are typically non-mucoid-smooth. Many organisms give mucoid colonies: some of the aerobic bacilli grown on a suitable medium, all the virulent pneumococci, virulent Friedländer bacilli, the nitrogen-fixing azotobacteria and

rhizobia and completely smooth strains of the mycobacteria, both acid-fast and non-acid-fast. The extracellular substance of some of these bacteria, like the pneumococci, stains as a definite capsule; of many others it does not. In the motion pictures capsulated organisms are to be distinguished from these others only by the fact that adjacent cells never come into contact—not even after long continued growth.

Concerning Bacterial Life Cycles

Some bacteriologists have imagined that structures often to be seen within bacterial cells play a part in a more or less complicated life cycle of these organisms. Examination in microculture offers a means of following what happens to these intracellular bodies. In no instance has there appeared evidence of nuclear material. The most commonly occurring structures within bacterial cells are dark appearing granules. Such granules commonly form at the ends of old rods of the colon-typhoid group of bacteria.[13] Cells in which they are just beginning to appear will grow, multiply and become internally homogeneous if they are planted on fresh medium. No development has ever been seen in bacteria with fully formed polar granules nor do the granules when they are freed from these old cells give any evidence of being alive. Dark granules are numerous in old filaments of such a bacillus as *B. megatherium* (Figs. 34–36). A cell containing a few of them will usually grow and multiply on fresh medium. . In this case the granules distribute themselves irregularly among the daughter cells which are otherwise perfectly homogeneous in appearance. If an old cell contains many of these granules it usually does not develop on fresh medium. Instead, it disintegrates, its membrane ruptures and the granules float away. Photographs made of them in this free state have given no reason to believe that they are alive. Many old mycobacterial rods are beaded. Slightly beaded rods become homogeneous again and grow normally on fresh medium; those which are strongly beaded have never shown evidence of being alive though they often rupture and free their granules. Much effort has been expended in attempting to prove that the azotobacterium (Figs. 58–62) has a

[13] See Fig. 4 in the article by Wyckoff.[6]

complicated life cycle.[14]   Old cells of this organism, which grows ordinarily as a large oval coccoid body, are shrunken and contain many dark granules (*B* of Fig. 58).   In their formation and subsequent history these old cells resemble the "resistant cells" of some yeasts. Planted on fresh medium they swell into normal cocci in which the granules are floating.   The subsequent behavior of these granules is the same as in *B. megatherium*, the protoplasm of most of the daughter cells being entirely structureless.   Because of the importance which has been attached to this organism in discussions of bacterial life cycles, a number of attempts have been made to reproduce its described pleomorphism.   On certain plate cultures rod-like forms have been found, apparently identical with air contaminants which grow luxuriantly on mannose media; aside from the development of its resistant forms, the azotobacterium itself was never pleomorphic.

The only other structures observed within bacteria have been seen in injured and disintegrating cells.   Old bacilli having fragile membranes (Figs. 44–45) and bacteria which are swollen through the action of LiCl or other reagents often vacuolate before they burst.   Some of these bacteria also contain irregular granular masses that appear to be bits of coagulated protoplasm.   When their cells rupture, this coagulated material is commonly extruded and can be seen free in the medium.   Nothing in its subsequent behavior in the motion pictures suggests that it is living.   Very possibly this cell débris is to be identified with the symplastic stage of the bacterial life cycles.

SUMMARY

Using a micro motion picture technique for making records, studies covering several thousand hours of observation have been made of the growth of a number of bacteria.   On the basis of these experiments a discussion is offered of bacterial division and its influence on gross colony appearance, of different kinds of pleomorphism that have been observed, and of the nature of the internal structure that is seen in some bacteria.   Several of the microorganisms chosen for examination are ones that have been thought to give evidence of life cycle phenomena.   The present pictures, however, contain no evidence of a bacterial cycle in the commonly accepted meaning of the term.

[14] Löhnis, F., *Mem. Nat. Acad. Sc.*, 1921, 16, 1, 2nd memoir.

EXPLANATION OF PLATES

PLATE 25

Magnification, 780 ×.

FIGS. 1–4. The development of a microcolony of a spore-bearing aerobe (*B. flexus* (?)) which normally splits by fission into bacilli of approximately equal size. Shortly after Fig. 4 was made all the bacteria suddenly became motile; after about 2 hours this motility quite as suddenly ceased.

FIGS. 5–7. Stages in the development of a microcolony of *B. lactis acrogenes*. This organism offers a perfect example of multiplication by fission. As the appearance of the microcolony of Fig. 7 suggests, the visible colonies are smooth (non-mucoid-smooth) in type.

FIGS. 8–11. Growth of *Staphylococcus aureus* in a heavily seeded micropreparation. Coccoid division is well illustrated by the groups *A* in Figs. 8 and 9 and *B* in Figs. 9–11. The swelling that precedes splitting is especially evident in *A*.

FIGS. 12–15. Details of the first two divisions in the development of a microcolony of *Micrococcus luteus*. Coccoid division in this organism gives rise to bacteria of unequal size.

PLATE 26

Magnification, 780 ×.

FIGS. 16–20. Stages in the growth of a microcolony of *Streptococcus pyogenes*. The organism *A* of Figs. 16 and 17 multiplies by true coccoid division. Many bacteria in the chains of Figs. 19 and 20 elongate and split by a process that is essentially fission.

FIGS. 21–24. The development of a microcolony of a non-acid-fast mycobacterium (Culture 23). The old coccoid elements of Fig. 21 sprout into filaments which in Figs. 23 and 24 show many branches budding from them.

FIGS. 25–27. Another growth of the same organism showing how the long branched filaments formed during the most active growth break up into rods as this stage passes.

FIGS. 28–29. The old coccoid forms of Fig. 29 arise by successive division from the rods of the preceding figure.

FIGS. 30–33. First divisions in the growth of a strain of *Streptococcus pyogenes*. The sprouting and incomplete parting of the bacteria *A* are like those seen among the corynebacteria. Somewhat later all divisions were typically coccoid.

PLATE 27

Magnification, 780 ×.

FIGS. 34–36. Bacilli from an old culture of *B. megatherium*. Most of the rapidly growing bacilli are long and filamentous. The very granular cells on the left of Fig. 34 did not develop nor did the granules freed from *A*.

FIG. 37. Rods and filaments of a *B. subtilis*-like spore-bearing aerobe. Extracellular granules like those at *B* have sometimes been described as examples of life

cycle forms. Followed by the camera they have never given evidence of being alive.

FIGS. 38–39. Development of a microcolony of *B. pyocyaneus*. Fig. 39 illustrates the wide variations in size of this organism. Contained within the bacterial mass of Fig. 39 are many long filamentous forms. This figure shows the geometrical pattern of which they form the groundwork and which is apparent in microcolonies of the two other pseudomonades that have been photographed. At the time these pictures were made the bacteria had pronounced motility with much drilling back and forth of single organisms.

FIG. 40. Pleomorphic bacteria in a growth of a strain of *B. subtilis* showing swollen forms (*A*), rounded bits of protoplasm extruded from ruptured cells (*B*) and terminal cocci (*C*). All these aberrant forms behave as if they were dead or were parts of degenerating cells.

FIG. 41. An early stage in the development of a microcolony of *N. catarrhalis*.

FIG. 42. The colony of Fig. 41 after further growth. The individual cells have become much smaller in size.

FIG. 43. Bacilli of *B. flexus* (?) cultivated on a glycerinated medium. These curled organisms are to be contrasted with the uniform straight rods on nutrient agar (*cf*. Figs. 1–4).

PLATE 28

Magnification, 780 ×.

FIGS. 44–45. Disintegration forms appearing in a culture of *B. subtilis*. Many of the faint rods of Fig. 45 are produced by the rupture of dark rods in the preceding picture. The spherical forms ("macrococci") are masses of protoplasm extruded by these dying or dead cells. The motion pictures illustrate all stages in the production of such a sphere *B* from the rupture of the long rod *A*.

FIGS. 46–53. Steps in the development of a microcolony of a very mucoid strain of the frog bacillus, *Mycobacterium ranae*. The bacterium at *A* multiplies by repeated branching. As in the succeeding pictures of mucoid growth the bacteria are held apart, either singly or in chains, by the voluminous and viscous products of their metabolism.

FIG. 54. A 24 hour growth of smooth Type II pneumococci. The effect of the capsule in holding the bacterial chains apart from one another is evident.

FIG. 55. A microcolony of a rough strain of pneumococci. Deprived of their capsular material, these bacteria lie closely packed together.
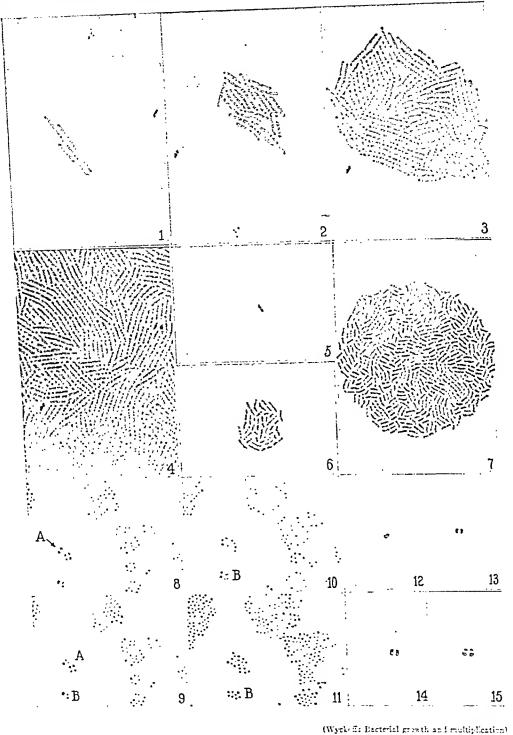
FIG. 56. A young growth from a frequently transplanted strain of smooth Type III pneumococci. The wide separation of the bacterial chains corresponds to the extreme mucosity of their growth. As the culture ages these streptococcoid chains become shorter and eventually are packed closer together.
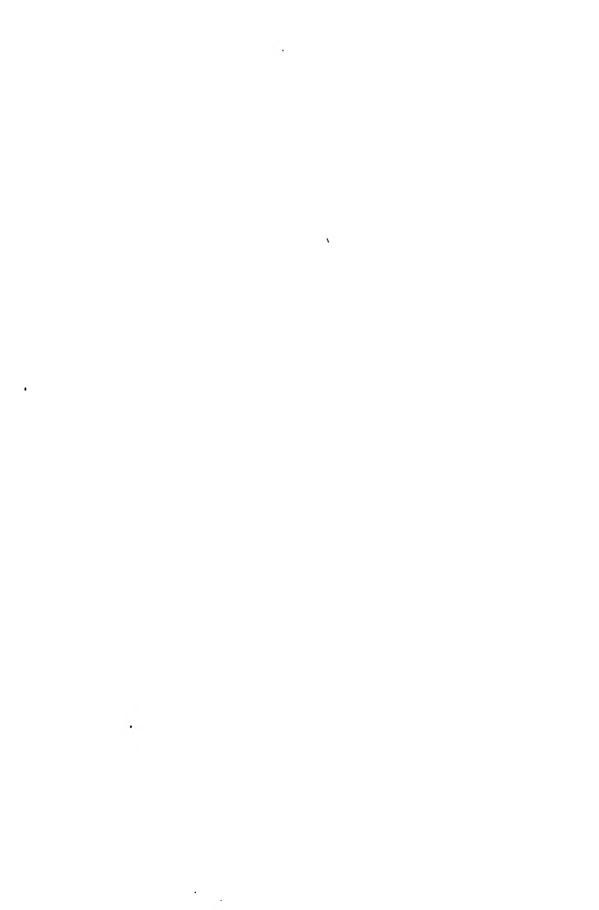
FIG. 57. A microcolony of the very mucoid *Rhizobium radicicola*. The jelly-like substance in which the individual bacteria lie imbedded is clearly evident.
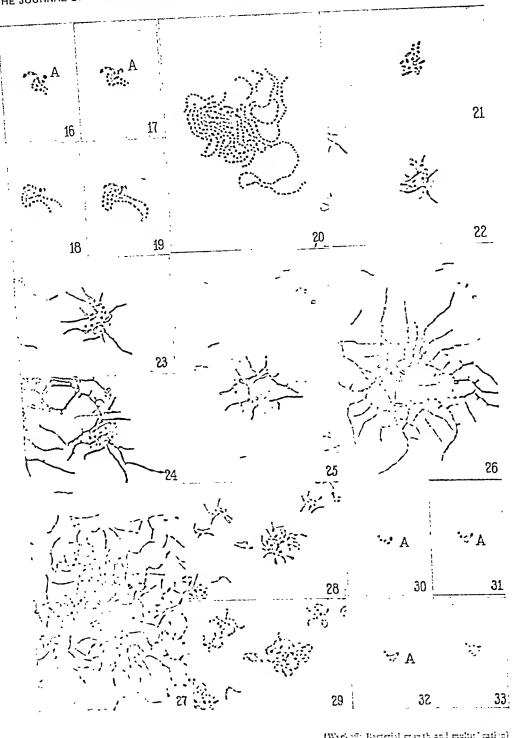
FIGS. 58–61. A growth of *Azotobacter beijerinckii*. The chain of diplococci *A*

of Fig. 61 arises from the pair A' of Fig. 58. The "resistant" cells B of Fig.
develop again into normal vegetative forms (B' of Fig. 61). The granules in
do not multiply.
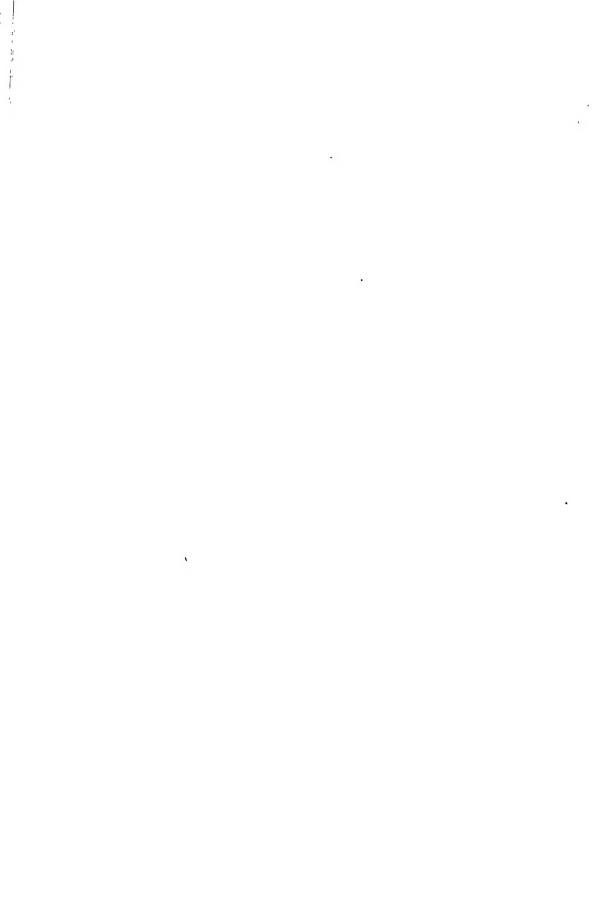
FIG. 62. An advanced growth of a "rough" strain of the turtle bacillus, My
bacterium chelonei. This picture suggests the way in which the heaped-up co
nies of the typically convoluted mycobacterium result from a packing togethe
long strands of intertwined bacilli.
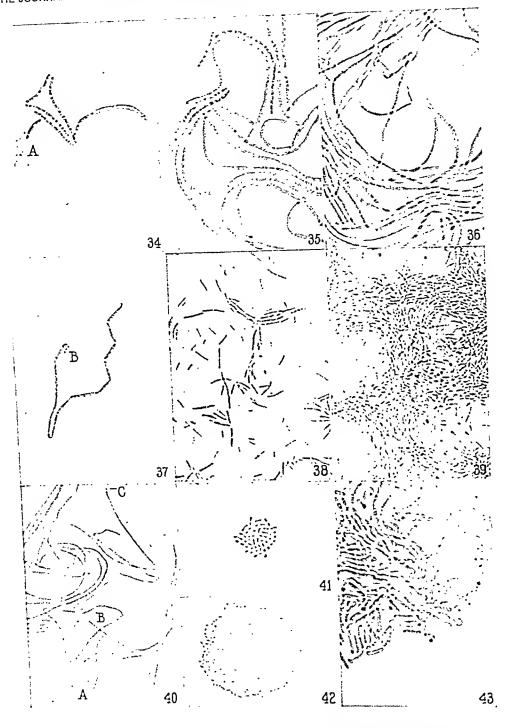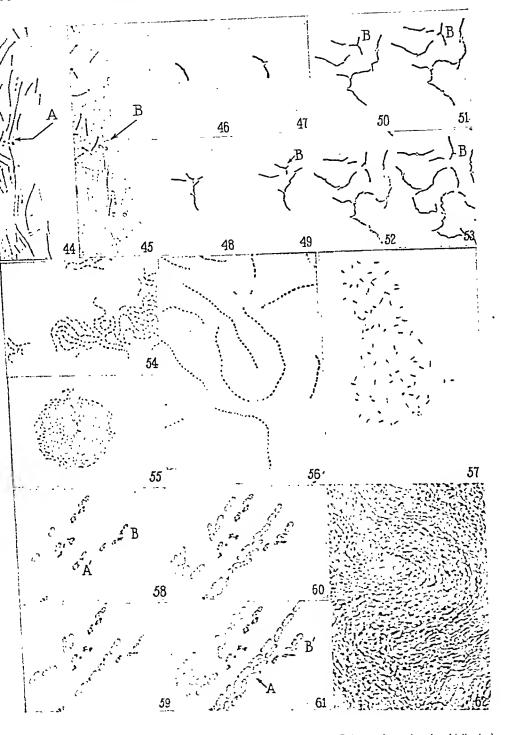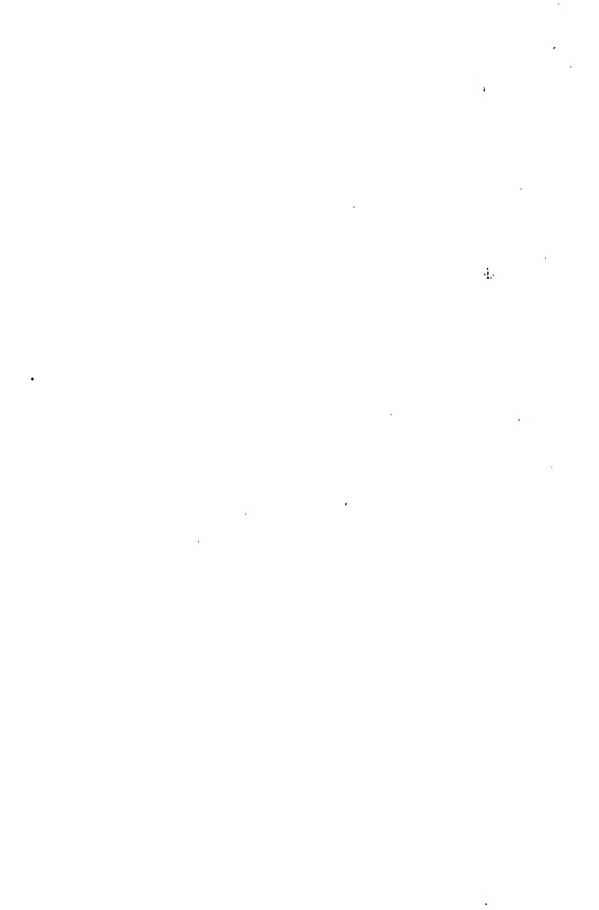
PLATE 25

PLATE 27

THE FILTERING CAPACITY OF LYMPH NODES

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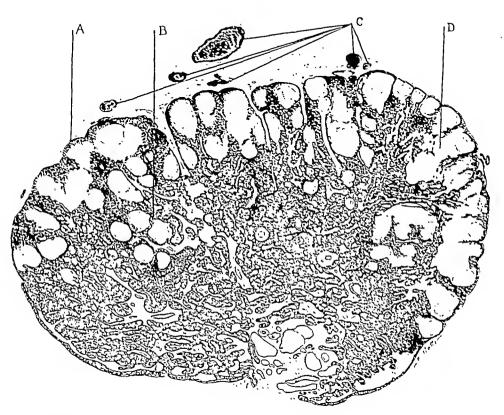
Lymph nodes have two obvious functions, the production of certain cells and the arrest of foreign material brought to them by the lymph. The second of these functions, that is, the filtering capacity of the nodes, has been the subject of a large amount of indirect experiment and speculation. It would be unprofitable and entirely repetitive to describe this work in view of the excellent reviews of Hellman (1) and Oeller (2).

The nodes are held to provide two sorts of filtration, the first of simple mechanical type and the second biological—due particularly to the phagocytic activity of the reticulo-endothelial cells. Opinions as to the combined efficiency of these two types—normal nodes alone being under consideration—vary from assertions of complete effectiveness to very much the reverse.

As a result of a great deal of experience with the lymphatics in the hind leg of the dog, it became relatively simple for us to perfuse the popliteal and iliac lymph nodes under conditions of pressure and flow normal for the animal and with the blood circulation completely intact. Prior to a description of typical experiments it will be well to review the architecture of a lymph gland. such as the popliteal node of the dog. together with what is known of the normal flow of lymph through it.

Text-fig. 1 is a drawing of a popliteal node injected with a dilute solution of India ink. The injection mass has been delivered through a cannula in one of the large trunks along the saphena parva vein. The injection pressure was 20 mm. of mercury. This is less than the

pressure which can be developed in the same vessel when the muscles of the leg are stimulated electrically. The injection enters the node through a number of afferent trunks which pierce the capsule obliquely and open into the marginal or cortical sinus. This sinus is not a channel but is a large bowl-shaped lake, bounded upon the outside by

TEXT-FIG. 1. Camera lucida drawing of a section of the popliteal lymph node of a dog injected through the afferent lymphatics with a dilute suspension of India ink. *A*, cortical sinus; *B*, intermediate sinus; *C*, afferent lymphatics in the capsule; *D*, lymphocytes. × 9.

the capsule of the node and on the inside by the lymphocytic parenchyma. The lake is traversed by fibrous trabeculae and blood vessels and, like all the sinuses, is crossed and recrossed by a fine mesh of reticulum most important for the filtering function of the node. From the cortical sinus irregular but numerous cleft-like channels, the intermediary sinuses, pass between the masses of lymphoid tissue

toward the hilus of the gland, where they unite with the cortical sinus to form the efferent lymph vessel. It is a fortunate circumstance for our experiments that this vessel is usually single at the hilus, thus permitting complete collection of all the lymph passing through the node.
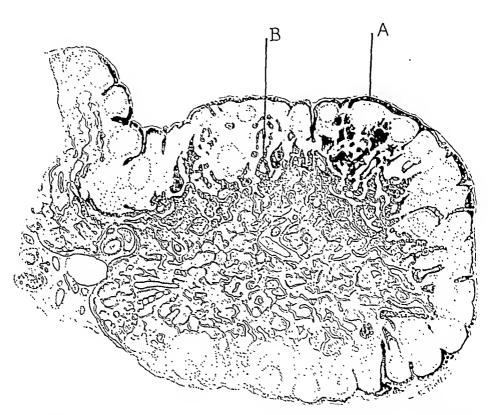
The architecture of the sinuses has a very direct relation to the filtering capacity of the node. Not only are they crossed and recrossed by the reticulum, but their walls are incomplete wherever lymphocytic growth is active (Drinker, Wislocki, and Field (3)). This means that at many points, particularly upon the inner surface of the cortical sinus, the lymph may run out into the lymphoid tissue.

Nordmann (4), as a result of study of sections of nodes, arrived at the conclusion that lymph traverses a node in three ways. Most of it reaches the efferent vessel without leaving the marginal sinus. A second fraction, much smaller, finds its way *via* the intermediary sinuses, and a third, very small part, drifts from the marginal sinus into the lymphoid tissue and finds its way back into the main stream either by rejoining the marginal sinus or by reaching an intermediary sinus. Maximow (5) adds another possibility, which we have not encountered; namely, an occasional endothelium-lined lymphatic which goes directly through the node. Such atypical vessels must be very infrequent and represent survivals of embryonic lymphatics which have remained free of lymphoid tissue.

In Text-fig. 1, the black injection mass fills the cortical sinus and is deposited upon the walls and reticulum of the intermediary sinuses. The masses of lymphoid tissue are but slightly penetrated by the ink, but the barrier to the entrance of the injection resides in the density of the lymphocytic accumulations rather than in an intact sinus wall.

Text-fig. 2 is a drawing of the iliac lymph node taken from the groin of the dog which provided the popliteal node seen in Text-fig. 1. The ink reaching the iliac node is that which passed through the popliteal node. It is clear that the marginal sinus is not entirely filled and that the ink has flowed into many intermediary sinuses. As a result of numerous injections it is our opinion that lymph entering the marginal sinus flows as readily through the intermediary sinuses as it does through the cortical sinus. The entire arrangement, from the point of view of mechanics, is excellent for filtration. Lymph flowing in

through a number of narrow channels, and under a very definite head of pressure, finds itself in a huge space with an enormous number of wide and irregular paths which lead to the hilus vessel. The flow is instantly slowed and the driving head of pressure practically lost. Not only are the sinuses in the node a perfect settling chamber, but

TEXT-FIG. 2. Camera lucida drawing of a section of the iliac lymph node taken from the dog which provided the popliteal node of Text-fig. 1. *A*, cortical sinus; *B*, intermediate sinus. × 13.

the reticulum which they contain furnishes a multitude of baffles which again slow lymph flow and make it easy for the phagocytic cells composing the reticulum to perform their function.

EXPERIMENTS

It is difficult to perfuse lymph nodes in rabbits and cats. One can collect small amounts of fluid from afferent and efferent vessels, but a steady experiment run-

ning over some hours is a hard task. In dogs the diameter and toughness of the vessels which must be cannulated, and the large size of the popliteal node, make the problem quite simple.

Two varieties of preparation have been employed, in both instances using dogs anesthetized with nembutal. In the first, after a subcutaneous injection of 2 per cent trypan blue in the foot, an incision is made at the lower end of the popliteal space. This discloses the afferent lymphatics. A small quartz cannula is tied in a single branch about 5 cm. below the node. All other afferents at the same level are ligated. The cannula is then filled with dilute trypan blue in physiological saline, and this is driven into the gland under low pressure. The incision is now carried up the leg and the popliteal lymph node uncovered. The efferent lymphatic is deeply embedded in fat and lies at the upper end of the node. Unfortunately, it is usually upon the anterior surface of the node and corresponding-ly awkward to cannulate. When both cannulas are in place the afferent line is filled with perfusate and connected to a small graduated reservoir containing the balance of the perfusing fluid. A constant head of pressure is provided by a small column of mercury adjusted by means of a levelling bulb.

In such a preparation as this, the lymph flow through the node is entirely isolated and one gets a volume of fluid from the efferent lymphatic which soon becomes equal to that delivered to the afferent vessel. The blood circulation need suffer no interference whatsoever.

In the second type of preparation the afferent lymphatic has been cannulated in exactly the same way. The thoracic and right lymphatic ducts were then isolated and the former cannulated. In order to make direct entrance of lymph into the circulation impossible, the precaution was taken to tie both subclavian veins central to the points of lymphatic entrance. In an animal so prepared, one has in the thoracic duct lymph a representation of what may reach the circulation after traversing the popliteal and iliac lymph nodes and the entire length of the thoracic duct.

As perfusion fluid, heparinized plasma from the dog employed in the experiment was usually the basic fluid. This was diluted with physiological salt solution until the protein content was approximately 1.0 per cent. To such artificial lymph, washed red corpuscles were added so as to give a count of approximately 25,000 per c.mm. This is a reasonably normal lymph, containing red cells from the dog under experiment as the particles to be filtered out. In a slow, non-pulsatile perfusion it is necessary to equip the perfusion reservoir with a stirring device in order to prevent sedimentation of red cells. When hemolytic strepto-cocci were used as particles, they were grown in a mixture of broth and 20 per cent dog serum, or in dog serum alone. In certain experiments the culture was em-ployed as perfusate without dilution; this being done to gain a graphic expression of the sites of arrest of the organism in the node and also to see whether, in the presence of great numbers of cocci, the organisms could be found in blood capil-laries within the perfused gland. When cultures were diluted with physiological saline, the final mixture contained approximately 1 per cent of dog serum protein.

Two strains of hemolytic streptococci were employed. One of these, known
Streptococcus 1, was isolated from a human case and was highly virulent for mic
but caused no reaction in normal dogs when injected into the blood stream, in
the lymph stream, and subcutaneously. The second strain, Streptococcus 2, w;
isolated from edema fluid collected from the leg of a dog in which the lymphati
had been completely obliterated. This animal had had eight attacks of chil
and fever similar clinically to those occurring in human beings with lymphedem
and elephantiasis. Early in these attacks, which occurred spontaneously,
streptococcus was invariably present. This organism causes a brief period (
fever when large amounts of culture are injected subcutaneously in normal dog
and a severe febrile reaction when injected into the lymphedematous leg of a do
with lymphatic obstruction. The organism is non-virulent for mice. So far a
filtration was concerned, no differences between the two organisms were notec
and the experiments cited are all concerned with Streptococcus 1.

Experiment 1. Dog, Weight 19.3 Kilos.—Perfusion of the popliteal node wit;
red cells from the dog used in the experiment. These cells were suspended in th
dog's own heparinized plasma diluted with physiological saline to contain 1.0(
per cent protein. Red cell count in the perfusate 26,400 per c.mm. Perfusio;
pressures 16 to 20 mm. Hg. In 2 hours and 5 minutes, 9 cc. of perfusate ran in
and 7.6 cc. were collected from the cannula in the efferent lymphatic. The tota
effluent was collected as 13 separate specimens. Of this number, 9 contained n(
red cells. 3 contained 200 red cells per c.mm., and 1 contained 400 red cells pe
c.mm. Filtration has been fairly complete. If in such an experiment the node
is massaged, even very gently, the effluent at once contains red cells in large
numbers.

On microscopic section, red cells were found in both cortical and
intermediate sinuses, but were most numerous in the latter. Many
were phagocytized by reticulo-endothelial elements, but the greater
number lay in closely packed masses scattered through the reticular
meshwork of the intermediate sinuses.

Experiment 2. Dog, Weight 18.7 Kilos.—Perfusion of the popliteal node
with an undiluted serum-broth culture of Streptococcus 1. Culture contained
600,000,000 colonies per cc. Perfusion pressure 34 mm. Hg. In 1 hour and 20
minutes, 5 cc. of the culture ran into the node and were collected from the efferent
lymphatic. Cultures of the entire effluent showed 4,500,000 colonies per cc.
Filtration was 99 per cent complete.

Fig. 1 is a camera lucida drawing of a section of this popliteal node.
Part of the cortical sinus is seen just beneath the capsule in the right
side of the illustration. The black material in this sinus, and in the
Y-shaped intermediary sinus, consists of masses of streptococci both

free and attached to cells.   Fig. 2 shows part of the marginal sinus just above a dense collection of lymphocytes into which the organisms have not penetrated.   This last is not invariably the case.   Regions were found where the lymphocytes were quite solidly packed, but here and there large mononuclear or, occasionally, polymorphonuclear cells containing cocci were seen.   Fig. 2 is a higher magnification of part of the cortical sinus.   When a gland is given such huge dosage as was the case in this experiment, blood cultures occasionally become positive, and this when the precaution has been taken to tie the thoracic and right lymphatic ducts and both subclavian veins.   The explanation apparently resides in the migration into blood capillaries in the node of phagocytes containing streptococci which are still capable of growing.   Fig. 3 shows a capillary in the loose tissue just outside the capsule of the perfused node of Experiment 2.   In addition to red cells, it contains 9 white cells containing microorganisms. Schulze (6) has summarized the literature and produced experiments to the effect that the capillaries in lymph nodes possess walls which are not solid, so that there is a direct communication between the blood and lymph.   If this were true, microorganisms might enter blood capillaries in nodes, provided some force could be found which would develop a current into the capillaries.   In the spleen, such a force is supplied by the smooth muscle in the capsule and trabeculae.   The pulsations of the organ drive fluid and cells back into the circulation, the latticed splenic capillaries being easy to enter.   We have been unable to produce contraction of the popliteal lymph node in the dog, nor have we found smooth muscle in the capsule or trabeculae.   If a node is perfused too vigorously, or if the outflow is obstructed, swelling is readily produced and on removal of the cause goes away slowly; not as would be the case if smooth muscle contracted, but as a gradual return expressing a poor degree of elasticity.

It is not easy to see just how a lymph node could retain structural integrity if it possessed open blood capillaries and no power of rhythmic contraction which might be counted upon to drive plasma and cells back into the blood vessels and clear the node of the excess transudate which must steadily accumulate.   Furthermore, if the capillaries in the popliteal node are open, then lymph collected from afferent vessels ought to contain much less protein than that from the efferent side. This is not the case.   Protein concentrations are identical.

Apparently the vascular system in the popliteal node is closed and ameboid activity is essential for entrance. This may not be the case with other nodes in the dog, and between different animals wide variations may exist. The subject is of real importance, since open capillaries in certain groups of nodes might account for high lymph protein, a finding often hard to explain on the basis of water absorption alone.

Experiment 3. Dog, Weight 21.2 Kilos.—Perfusion of the popliteal node with a diluted dog serum-broth culture of Streptococcus 1. Organism an 18 hour culture. Dog serum obtained from the animal used in the experiment. Perfusate contained 1.08 per cent serum protein. At the start of the experiment the perfusate contained 5,000,000 colonies per cc., and at the close 30,000,000 colonies. This multiplication is unavoidable under the conditions of warmth which must obtain during the experiment.

Afferent and efferent vessels of the right popliteal node cannulated in the usual way, and connection with the perfusion apparatus established. In order to be certain that the gland was entirely isolated the thoracic duct was cannulated and all lymphatic entrances into the right and left subclavian veins were tied. Perfusion pressures 20 to 30 mm. Hg. In 1 hour and 18 minutes, 7 cc. of perfusate ran into the node, and 6.1 cc. were collected from the efferent lymphatic. The results as regards filtration are expressed in Table I. No explanation can be given for the sterility of the last specimen of effluent. In this instance the blood remained sterile and no organisms were found in the thoracic duct lymph.

On microscopic section the node seems to have been slightly stretched and edematous. Organisms can be found only after long search and always attached to endothelial cells in the reticular framework of the cortical sinus.

Experiment 4. Dog, Weight 21 Kilos.—Perfusion of the popliteal node with a diluted culture of Streptococcus 1. Organism an 18 hour serum-broth culture. Serum obtained from the animal used in the experiment. Perfusate contained 1.4 per cent protein. At the start of the experiment the perfusate contained 300,000 colonies per cc.; at the close, 500,000 cc.

Preparation the same as in Experiment 3. Perfusion pressures 14 to 24 mm. Hg. During 60 minutes, 10.2 cc. of perfusate ran into the gland and 9.6 were recovered. The results as regards filtration are given in Table II.

When removed, the node did not appear distended, but on microscopic examination the sinuses were possibly overwide. Organisms were found on reticular strands and in the endothelial cells of the reticulum.

Experiment 5. Dog, Weight 23.5 Kilos.—Perfusion of the popliteal and iliac lymph nodes with an undiluted serum culture of Streptococcus 1. Organism a 1½ hour culture in serum taken from the animal used in the experiment. At the finish of the perfusion the perfusate contained 250,000,000 colonies per cc. Perfusion pressures 20 to 40 mm. Hg.

The right lymphatic entrances into the subclavian vein were tied. The thoracic

duct was cannulated in the neck and all lymph excluded from veins on the left side. As a final precaution the subclavian veins were tied central to the observed lymphatic entrances. The afferent lymphatic of the left popliteal node was cannulated in the usual way. Efferent vessels of the node were untouched. This experiment utilized Preparation 2, and was designed to indicate the degree of filtration accomplished by two nodes, together with the possible settling effect

TABLE I

Perfusion Flow and Figures for Filtration of Streptococci in Experiment 3

Time	Perfusion inflow	Perfusion inflow per min.	Perfusion outflow	Colonies per cc.
min.	*cc.*	*cc.*	*cc.*	
0–16	2.0	0.13	1.5	Sterile in 0.4 cc.
16–36	2.0	0.10	1.8	100 colonies per cc.
36–57	2.0	0.10	1.8	2500 colonies per cc.
57–78	1.0	0.05	1.0	Sterile in 0.4 cc.

Perfusate at start 5,000,000 colonies per cc.
Perfusate at finish 30,000,000 colonies per cc.
Thoracic duct lymph after 46 minutes perfusion of node, sterile in 0.4 cc.
Blood cultures taken after 4, 48, and 75 minutes of perfusion, all sterile.

TABLE II

Perfusion Flow and Figures for Filtration of Streptococci in Experiment 4

Time	Perfusion inflow	Perfusion inflow per min.	Perfusion outflow	Colonies per cc.
min.	*cc.*	*cc.*	*cc.*	
0–15	1.7	0.11	1.7	15,000
15–30	2.7	0.18	2.3	30,000
30–45	3.1	0.20	2.9	60,000
45–60	2.7	0.18	2.7	120,000

Perfusate at start contained 300,000 colonies per cc.
Perfusate at finish contained 500,000 colonies per cc.
Thoracic duct lymph after 27 and 64 minutes of perfusion, sterile.
Blood culture taken after 68 minutes of perfusion, sterile.

which might occur in the long flow through the thoracic duct. The results are summarized in Table III. In this case few organisms reached the thoracic duct, and even if the duct had been allowed to empty into the subclavian vein it is doubtful whether positive blood cultures would have been obtained.

At the close of the experiment, 3 cc. of 2 per cent trypan blue were sent in

through the perfusion cannula. This blue appeared promptly in the thoracic duct lymph. Both the popliteal and iliac nodes were deeply and uniformly stained, and neither showed evidence of edema either grossly or microscopically.

On section of the popliteal node, occasional organisms were found free in the cortical sinus. Most of them were attached to cells, frequently to polymorphonuclear leucocytes which were numerous all through the node sinuses. The large reticular cells were seen in the dense groups of lymphocytes. The iliac node showed a large number of polymorphonuclear leucocytes in the sinuses, particularly at the periphery of the node, but on long search no cocci were found.

TABLE III

Perfusion Flow and Figures for Filtration of Streptococci in Experiment 5

Time	Perfusion inflow	Perfusion inflow per min.	Thoracic duct lymph Colonies per cc.
min.	*cc.*	*cc.*	
11.40 a.m.			Control specimen sterile in 1.0 cc.
0–13	2.8	0.22	Sterile in 1.0 cc.
13–29	3.0	0.19	Sterile in 1.0 cc.
29–43	2.0	0.14	Sterile in 1.0 cc.
43–56	2.0	0.15	Sterile in 1.0 cc.
56–71	2.0	0.14	Sterile in 1 drop. Streptococci found in 1 cc.
71–88	2.2	0.13	Sterile in 1 drop. Streptococci found in 1 cc.

Blood cultures, taken before perfusion and 25, 41, 73, and 89 minutes after perfusion, were sterile. At the finish of the perfusion the perfusate contained 250,000,000 colonies per cc.

In this instance, in which thoracic duct lymph is used to indicate filtration, it is clear that a high degree of efficiency has been obtained.

Experiment 6. Dog, Weight 24.5 Kilos.—Experiment in every way similar to Experiment 5, except that a higher perfusion pressure was used, 40 to 50 mm. Hg instead of 20 to 40 mm. Hg. The results are summarized in Table IV. The perfusion at the close of the experiment contained 300,000,000 colonies per cc.

In this case filtration has not been so successful, due in all probability to the increased pressure and uniformly higher rate of flow. On removal, the popliteal and iliac lymph nodes were not distended. The cortical sinus of the popliteal node contains many cocci and these are found through the intermediate sinuses all the way to the hilum of the gland. Many of the organisms are free. About an equal number are

attached to reticular strands or polymorphonuclear leucocytes.   Cocci can be found in capillaries both free and in phagocytes.   The iliac node shows many organisms, almost as heavy a load as in the popliteal gland, and in the same situations.

In this perfusion the first node was evidently quite inadequate, but the second in the line has been fairly successful in blocking the organisms and this in the face of a rapid flow of a perfusate heavily loaded with streptococci.

TABLE IV

Perfusion Flow and Figures for Filtration of Streptococci in Experiment 6

Time	Perfusion inflow	Perfusion inflow per min.	Thoracic duct lymph Colonies per cc.
min.	*cc.*	*cc.*	
12.59 p.m.			Control specimen sterile in 1.0 cc.
0–15	3.3	0.22	Sterile in 1.0 cc.
15–30	2.9	0.19	60 colonies per cc.
30–45	3.1	0.20	1,000,000 colonies per cc.

A final thoracic duct lymph specimen, taken 15 minutes after ceasing perfusion, contained 7,000,000 colonies per cc.   Blood cultures, taken 20, 41, 55, and 70 minutes after perfusion began, were sterile.   A final culture at 82 minutes showed streptococci.   At the finish of the perfusion the perfusate contained 300,000,000 colonies per cc.

DISCUSSION

Several points are of importance in considering the actual significance of these experiments.   It has been shown that the large afferent lymphatics in the leg of the dog will support pressures as high as 81.0 mm. Hg (Field, Drinker, and White (7)).   Such pressures as this were obtained as a result of sterile inflammation of the foot.   In an afferent lymphatic just below the popliteal gland, one of the vessels utilized in our perfusion experiments, pressures of 50 mm. Hg have been observed during repeated passive motion of the foot.   It is thus clear that in normal animals moving about actively, lymph pressures equalling those in our perfusions may occur.   Measurements of the flow of lymph from an afferent lymphatic below the popliteal node in walking and running dogs (White, Field, and Drinker (8) and Weech, Goettsch, and Reeves (9)) show that such rates of flow as have been used in our

perfusions are not abnormal. In a quiescent dog the flow of lymph from such vessels soon becomes negligible and the lymph pressure falls to zero. If one considers the degree to which lymph nodes might filter organisms under actual conditions of disease, it is apparent that they would be subjected to a much less severe task than that imposed by our experiments. Given a cellulitis of the foot, the dog is ordinarily quiet and a heavy flow of lymph would not occur until swelling became extreme. It is also certain that lymph nodes in actual disease would rarely be confronted with such a deluge of organisms as has been placed directly in the lymphatics in our experiments.

The perfusions cited in this paper are typical examples of thirty-five experiments. They indicate that lymph nodes, even under extreme conditions, possess a high degree of filtering efficiency. Efforts were made to determine how far this is mechanical and how far biological. Streptococcus 1, grown on serum-broth in an 18 hour culture, was not encapsulated. When grown in dog serum for $1\frac{1}{2}$ hours, capsules were apparently present. In this second condition the cocci were less readily phagocytized by the dog's leucocytes, but perfusion of both types of organism showed no certain differences, though such an experiment as that summarized in Table IV does indicate a rather large escape of an encapsulated organism which was compelled to drift through two nodes.

There is a final point of general interest upon which these experiments throw possible light. It has long been a question as to whether lymph leaving a region of subcutaneous drainage can reach the blood stream without passing through a lymph node. In such experiments as Nos. 5 and 6—and eight of these were made—the thoracic duct lymph never showed an immediate deluge of cocci such as might be expected if nodes were short-circuited by vessels passing around them. Apparently lymph from peripheral regions does not reach the blood until at least a single node has been passed. This may not be invariable, but certainly in our experience it was the rule.

Finally, our facilities for work have not permitted us to test the filtration of microfilariae. Judging from the results with red cells, where filtration is complete, such large elements as the filariae would have great difficulty in passage. It is our hope that this paper may cause workers in the tropics to try such an experiment.

SUMMARY

In anesthetized dogs the popliteal lymph node alone, and the popliteal and iliac lymph nodes in series, have been perfused with solutions containing dog erythrocytes and streptococci. The perfusions have been carried out under conditions of lymph flow and pressure within the limits of those occurring in the actively moving dog, or after a severe degree of inflammatory swelling has developed. Figures for filtration are given, with protocols of typical experiments. They indicate that normal lymph nodes possess a high degree of filtering efficiency—an efficiency so great as to make it fairly certain that in a part kept at rest early in an infection, practically no microorganisms would escape the nodes in the line of drainage.

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EXPLANATION OF PLATES

PLATE 29

FIG. 1. Camera lucida drawing of a section of the popliteal lymph node perfused with a serum-broth culture of Streptococcus 1 in Experiment 2. A portion of the capsule of the node is seen on the right. Dark masses are streptococci caught in the sinuses of the node. Gram-Weigert stain. × 139.

PLATE 30

FIG. 2. Camera lucida drawing of a part of the cortical sinus in the popliteal node of Experiment 2. Gram-Weigert stain. × 683.

FIG. 3. Camera lucida drawing of a capillary from the node of Experiment 2. Note the phagocytes containing microorganisms. Gram-Weigert stain. × 1300.

PLATE 29

FIG. 1

PLATE 30

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FIG. 2

FIG. 3

(Drinker et al.: Filtering capacity of lymph nodes)
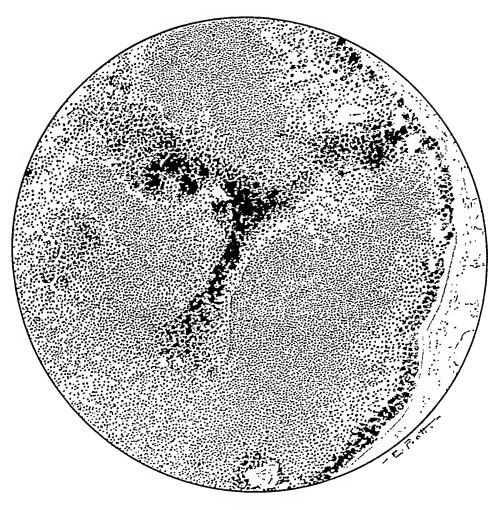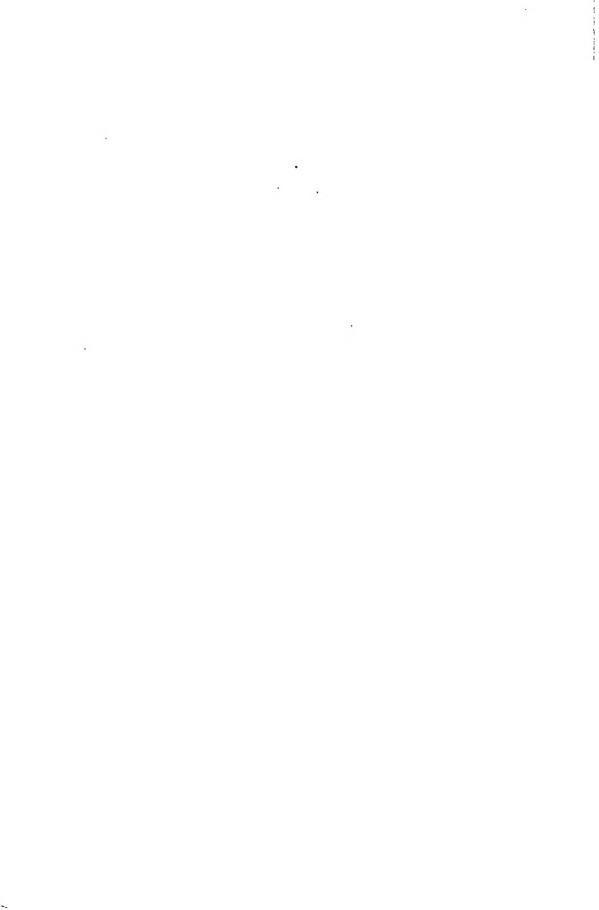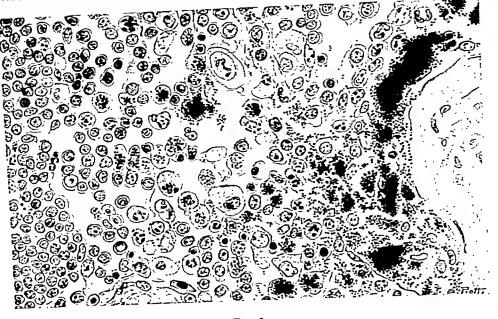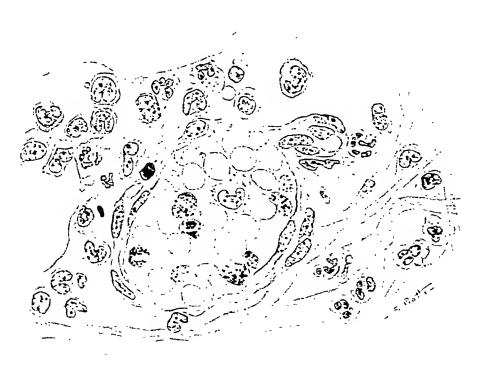
I. CHOLESTEROL AND CHOLESTEROL ESTERS IN DOG BILE

QUANTITATIVE METHODS

By ANGUS WRIGHT, M.D.

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In connection with other studies made in this laboratory on the constituents of the bile, with new methods available, it seemed wise to re-examine the whole question of cholesterol elimination in the bile. Method difficulties have impaired the value of many previous studies of biliary cholesterol. Recently, Elman and Taussig (6) and Andrews and Hrdina (1) have described methods of great similarity which are accurate for the quantitative analysis of biliary cholesterol.

The need of another method grew out of a series of experiments in which it was desired to determine the occurrence of *esters* of cholesterol in bile. In the methods (1, 6) mentioned, treatment with alkali is utilized to "fix" the bile pigments, and as such treatment would cause hydrolysis of any combined lipoids, these methods could not be used in the determination of esterified cholesterol. The occurrence of cholesterol esters in bile is a point about which there are differences of opinion. Thannhauser (9) states that all cholesterol in human bile occurs in a free state due to the action of an ester-splitting enzyme, "cholesterolesterase," which sets free the esterified cholesterol. Thomas (10) is quoted to the effect that cholesterol occurs in dog bile as an ester.

A large colony of closed, sterile bile fistula dogs of the type described by Rous and McMaster (8) furnished ample material for investigation.

Methods

1. The colorimetric method used for the determination of the total cholesterol in bile is that of Elman and Taussig (6) which is described in detail by them. At

the suggestion of Dr. W. R. Bloor we have used a red glass filter in the colorimeter when making these quantitations. This filter is of the type Corning No. 10, signal red (which transmits 96 per cent at 700 Å.u. and 0.0 per cent at 595 Å.u.). The filter affords the advantage of a much easier color match and consecutive readings on the same specimens check much more closely with the filter than they do without it. Also the filter eliminates the difficulty sometimes encountered when the extract is faintly brown. This method has served our need for determination of the total cholesterol in bile satisfactorily and duplicates checked within small limits.

2. For the determination of the *esters of cholesterol in bile* we have used a colorimetric method which is a modification of the well known Bloor (2) and Bloor and Knudson (5) methods for the determination of blood plasma cholesterol. This method as applied to bile, consists of running 5–10 cc. of bile (the volume of bile used depending upon the cholesterol content) slowly into 75 cc. of a mixture of 1 part ethyl ether and 3 parts of 95 per cent alcohol in a 100 cc. volumetric flask, shaking the flask vigorously at the same time to insure the formation of a fine precipitate. The mixture is then slowly brought to the boiling point, cooled, made up to 100 cc. with alcohol-ether mixture and filtered through a No. 2 Whatman filter paper. This extract keeps indefinitely if stoppered tightly and placed in the dark. Aliquots of the extract are taken, an excess of an alcoholic solution of digitonin added (2 cc. of a 0.50 per cent solution usually suffice) and the mixture is taken to dryness on the steam bath. The residue is extracted 3 times with petroleum ether (the fraction boiling off below 60°C.), using 20 cc. each time and boiling the solvent down to about 10 cc. The extract is then filtered with gentle suction through a sintered glass filter of the type marked "4G4 Schott and Gen. Jena." The filtrate is evaporated to dryness, extracted with chloroform and the color developed as in the method for total cholesterol. Pure cholesterol having a melting point of 145°C. is used as a standard. Two concentrations of standard are used, one containing 1.0 mg. per 5 cc., and the other 0.5 mg. per 5 cc. This method has been checked by adding esterified cholesterol in blood plasma to bile, with satisfactory recovery.

3. As a control over the above methods, the oxidative digitonin method of Okey (7) and Bloor (3) as modified by Yasuda (11) was used. We are grateful to Mr. P. L. MacLachlan of the Department of Biochemistry, who very kindly made the great majority of the digitonin determinations.

Comparison of the Colorimetric Method with the Oxidative Digitonin Method for Total Cholesterol in Bile

The results of these determinations are shown in Table 1. The colorimetric method gives results which run consistently 20 per cent higher than the digitonin method. These figures are in accord with those of Bloor (4) who has made a similar comparison of the colorimetric and digitonin methods as applied to blood plasma over a long

series of determinations and has expressed the opinion that the colorimetric values more closely approximate the true values. Our figures are at some variance with those of Elman and Taussig (6), who in a series of four determinations showed the colorimetric determinations to run sometimes greater and sometimes less than the digitonin. In our opinion, the variation in the digitonin results recorded by these workers can be largely, if not entirely, explained by the fact that the method of Okey (7) is not as uniformly accurate as it is in the form as modified by Yasuda (11).

TABLE 1

Comparison of Colorimetric and Oxidative Digitonin Methods Applied to Dog Bile

No.	Colorimetric	Digitonin	Colorimetric higher values than digitonin
	mg.	*mg.*	*per cent*
1	1.12	0.87	22
2	1.00	0.79	21
3	1.13	0.90	20
4	1.28	1.05	18
5	1.19	0.93	22
6	1.23	0.99	20
7	0.80	0.64	20
8	0.64	0.54	16
9	0.80	0.64	20
10	0.71	0.61	22
11	0.82	0.64	22
12	0.67	0.52	22
Average...			20

Just why the colorimetric method should run so consistently 20 per cent higher than the digitonin method is rather perplexing. Determinations of pure cholesterol and cholesterol added to bile of known concentration yield by the colorimetric method about 99 per cent of the theoretical values, whereas the digitonin method yields about 7 per cent less than the theoretical. It is possible that sterols related to cholesterol occurring in bile exert an influence in the development of a color which is not evident in the oxidative determinations.

The Physical State of Cholesterol Occurring in Normal Dog Bile

A series of six duplicate determinations on samples of bile from several healthy closed bile fistula dogs were made by the colorimetric and oxidative digitonin methods. The total cholesterol content of these bile specimens ranged from 0.190 mg. to 0.895 mg. In no instance was there evidence of esterified cholesterol in amounts permitting quantitation. From these results it is evident that in dog bile, as in human bile, cholesterol occurs entirely in an uncombined form.

CONCLUSIONS

The colorimetric method for the determination of total cholesterol in dog bile is consistently accurate as checked by the oxidative digitonin method. This method has the further advantages of being simple, rapid and economical.

A method for the determination of esterified cholesterol in bile is described and it is shown that there are no esters of cholesterol in normal dog bile.

We are indebted to Professor W. R. Bloor and Mr. P. L. MacLachlan of the Department of Biochemistry for their helpful advice and assistance in this work.

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II. BILE CHOLESTEROL

Fluctuations Due to Diet Factors, Bile Salt, Liver Injury and Hemolysis

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The blood and body fluids are so crowded with "chemical messengers" and vitamines that to some readers it appears a miracle that these substances ever reach their destination. Cholesterol has been looked upon as an innocent bystander, inert and going along with the crowd. Some of the recent work with hormones and vitamines would seem to focus attention on cholesterol as a close relative of other sterols and perhaps of ergosterol and the group of fat soluble vitamines. Further work with hormones (estrin and the male hormone) indicates a chemical constitution relating these "messengers" to the sterols. The same four ring nucleus is common to all these substances (18)

Therefore instead of an innocent bystander cholesterol may prove to be a messenger of importance and authority related to many vital body processes.

It can be seen from the tables below that cholesterol is influenced profoundly by bile salt metabolism and circulation. Bile salt feeding together with cholesterol may give maximal values for cholesterol in the bile. All evidence (15) points to the liver cell as the only source of bile salts but this does not necessarily mean that cholesterol is produced in the liver cell. However, it would be difficult indeed to prove

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that the liver is not concerned with cholesterol metabolism and its production in the body.

It is significant that the blood plasma of the dog contains 10 to 20 times as much cholesterol per 100 cc. as does the bile. Cholesterol in blood plasma averages 150–300 mg. per 100 cc. in contrast to bile which averages 10–15 mg. per 24 hour output in a total volume of 80–130 cc. This suggests a liver threshold of elimination but if such a threshold does exist it differs conspicuously from the renal threshold as it is understood today. It is possible to raise the blood cholesterol without a large increase in bile cholesterol and also to increase the cholesterol elimination in the bile without a change in blood cholesterol concentration. Cholesterol esters make up a large part of the blood cholesterol but the esters do not appear in the bile under the conditions of these experiments. The normal liver cell if it has a threshold for free cholesterol will not pass on into the bile any cholesterol esters. This question is receiving further study.

It may be argued that cholesterol as it appears in the bile is dependent upon the circulation of the bile salts. This may be in part a physical relationship as bile salts increase the solubility of cholesterol in the whole bile. It is also possible that the bile salts exert an influence upon the liver cell, modifying its physiological state and permitting the passage of cholesterol. It is generally accepted that the bile salts modify definite body functions in the gastro-intestinal tract in the external sector of their cycle in the body. We believe that the *internal sector of the bile salt cycle* may be even more important for the body, and that the hepatic cells and other body cells may be modified in their activity by the presence of bile salts. Interesting types of intoxication which develop in the fistula dog after long periods of bile salt deprivation point in this direction.

There is no dearth of experimental observations dealing with bile cholesterol in humans and animals. McMaster (9) has reviewed the earlier work and points out that much of the recorded data was unsatisfactory because of the type of bile fistula used. He showed that cholesterol in the bile can be increased by diets rich in cholesterol. The bile fistula introduced by Rous and McMaster (10) enables the investigator to collect accurately the 24-hour sample of sterile bile and marked a distinct advance in this field of study. Methods for bile cholesterol analysis have been unsatisfactory and inaccurate until quite recently and the recorded data are therefore inaccurate and subject to review. In the method used by McMaster

(9) the bile pigments introduce large errors and his base line for bile cholesterol output in the dog runs about double the amount recorded in the tables below.

Biliary cholesterol has been studied by D'Amato (2), Stepp (17), Dostal and Andrews (3), Fox (5), Salomon and Silva (16), Gardner and Fox (6), Elman and Taussig (4), McClure (8) and many others. Some of these papers deal with human, others with animal bile. The objections noted above apply to these observations. The greatest diversity of opinion on all phases of the subject is revealed by these papers.

Methods

The methods used in the quantitative determination of bile cholesterol are described above (Paper I). The bile fistula dogs were prepared according to the method of Rous and McMaster (10). Meticulous attention to the details of aseptic technique is needful in the care and daily bile collection in these animals (12). Their general supervision requires the bulk of the time of one technician. This type of fistula is made with excision of the gall bladder and insertion of a cannula in the common bile duct so that the bile is collected in a sterile bag. A comfortable canvas binder retains the bag and enables the dog to live a quiet and comfortable life for many months. It is highly important that these dogs remain in excellent clinical condition with little or no loss of weight and freedom from gastro-intestinal disturbances. Little significance can be attached to observations on dogs showing clinical abnormalities which are usually recorded in the published experiments from many laboratories.

The standard or control diet consists of a bread prepared in the laboratory and much used in the anemia colony. The bread contains wheat flour, starch, bran, sugar, cod liver oil, canned tomatoes, canned salmon, yeast and a salt mixture. Its preparation has been adequately described (20). This is a complete diet for the normal dog and will maintain anemic animals in health indefinitely. The *control periods* given in the tables below *precede* immediately the periods dealing with special diets, liver injury, bile feeding, etc. After operation there may be fluctuations in bile cholesterol which may be due to obscure factors. For this reason the dog was observed for a period of 7–10 days before the regular control periods were begun.

EXPERIMENTAL OBSERVATIONS

Brief clinical histories of the several bile fistula dogs are given in the following paragraphs. It will be noted that the weight at the end of the experimental observations is in no instance lower than the weight recorded at the beginning. This means excellent clinical condition, good food consumption and no gastro-intestinal disturbances. Fasting or intoxication will always reduce the normal output level of bile cholesterol.

Clinical Histories

Dog 32-161. Adult female white bull mongrel, operation Jan. 12, 1933. Weight at beginning of analyses 14.4 kg. Hemoglobin 144 per cent. This animal's weight remained constant except during a period of liver injury due to intravenous injection of hematin (Table 27) when the weight dropped to 13.0 kg. This loss was rapidly recovered and at present (Oct. 6, 1933), hemoglobin 111 per cent, the animal weighs 14.8 kg. The hemoglobin level has maintained a similar constant level with the exception of periods during which it has been lowered as a result of direct experimentation. Food consumption has always been good. The animal has been in excellent physical condition throughout the period of observation.

Dog 31-27. Adult female long haired mongrel, operation May 1, 1932. Weight 13.2 kg. Hemoglobin 119 per cent. Weight and hemoglobin have shown slight variation. Food consumption excellent. The animal has been exceedingly lively. On May 29, 1932, bile became infected and dog killed under anesthesia. Weight 13.7 kg., hemoglobin 105 per cent.

Dog 31-331. Adult female hound mongrel, operation June 1, 1932. Weight 16.9 kg. Hemoglobin 132 per cent. The hemoglobin showed slight variation and there was slight gain in weight to 17.4 kg. The animal was lively and consumed all of its food. On Dec. 21, 1932, the bile became infected and the animal was killed under anesthesia. At this time the weight was 17.4 kg. and the hemoglobin 105 per cent.

Dog 31-203. Adult female setter mongrel, operation Jan. 26, 1932. Weight 15.0 kg. Hemoglobin 107 per cent. The weight increased somewhat over a long period of experimentation, rising as high as 17.5 kg. The animal was in excellent physical condition throughout and consumed its food completely. The hemoglobin varied somewhat, going as low as 67 per cent during some of the experimental periods. The bile became infected June 3, 1933, and the animal was subsequently killed under anesthesia. At this time the animal weighed 15.9 kg., hemoglobin 93 per cent.

Table 21 gives characteristic control observations on two bile fistula dogs. Dog 32-161 shows a normal level in the first control period but a low normal in the second control period which was 7 months subsequently and followed a period of liver injury (see Table 27). The fore periods on salmon bread immediately preceded the test periods on calves' brains. In the control periods the fluctuations in bile cholesterol output from day to day rarely exceeds 1–2 mg. (see Table 25).

Calves' brains in the older experiments reported in the literature were usually fed with egg yolk and assumed to be in part responsible for the bile cholesterol increase if any was observed. In our experi-

ments the calves' brains alone (containing approximately 1.5 gm. cholesterol) have a negligible effect as a 10 per cent increase is within physiological fluctuations related to uncontrollable factors. The significant rise in bile cholesterol output when bile salt is added will be discussed under Table 23.

We know of no satisfactory explanation for the observations (Table 21) that the feeding of cholesterol in calves' brains gives no increase in biliary cholesterol, whereas the egg yolk feeding will give a definite increase (Table 23). It has been suggested (11) that the presence of the phosphatides and cerebrosides may prevent the absorption of the brain cholesterol.

TABLE 21

Bile Cholesterol and Calves' Brains Feeding

	Control diet—salmon bread			Control diet + calves' brain 230 gm. daily				
Dog No.	Duration	Bile volume daily average	Cholesterol average daily output	Duration	Bile salt fed	Bile volume daily average	Cholesterol average daily output	Cholesterol increase
	days	*cc.*	*mg.*	*days*	*gm.*	*cc.*	*mg.*	*per cent*
32-161	7	129	12.6	4	0	130	14.3	15
32-161	6	86	7.9	8	3	145	17.2	118
31-331	3	115	10.0	4	0	133	10.8	8
31-331	7	125	12.8	4	0	146	13.1	10

Table 22 shows some satisfactory and representative experiments with widely different food factors all done on the same dog which was in perfect physical condition and ate all the food as indicated. The sugar diet and zein (digested with trypsin) were given daily by stomach tube.

The control salmon bread diet periods show a large bile elimination—an average of about 140 cc. daily, and a uniform output of bile salt—an average of about 1.1 gm. per day.

Liver added to this control diet causes little or no change in bile volume, cholesterol or bile salt output. *Lean beef* feeding causes a distinct rise in bile salt but not in the cholesterol output. *Sugar* alone fed to a bile fistula dog always causes a sharp drop in bile volume and bile salt output. The drop in bile cholesterol is less conspicuous.

TABLE 22

Bile Cholesterol and Food Factors

Dog 31-203.

Control diet—salmon bread					Animal nnd grain protein and sugar				
Duration	Weight	Bile volume daily average	Bile salt daily average	Cholesterol average daily output	Duration	Diet—daily	Bile volume daily average	Bile salt daily average	Cholesterol average daily output
days	kg.	cc.	mg.	mg.	days		cc.	mg.	mg.
11	16.3	120	1078	14.1	14	Salmon bread + pig liver 300 gm.	109	1189	14.2
8	15.7	149	1228	12.0	6	Beef cooked 680 gm.	134	1510	13.0
9	16.0	154	1142	13.5	4	Sugar 50 gm.	67	523	8.3
9	17.3	139	1219	14.9	3	Zein 50 gm.	91	501	5.4

TABLE 23

Bile Cholesterol Influenced by Egg Yolk and Bile Salt Feeding

Dog No.	Weight	Duration	Bile volume daily average	Cholesterol average daily output	Duration	Yolks daily	Bile salt daily	Bile volume daily average	Cholesterol average daily output	Cholesterol increase
	kg.	days	cc.	mg.	days		gm.	cc.	mg.	per cent
31-27	13.4	7	110	10.5	6	4	0	109	15.0	50
31-27	13.3	6	123	10.3						
31-331	17.0	6	132	13.5	3	6	0	148	18.6	39
31-331	16.4	5	129	12.5	3	6	1*	171	19.8	46
31-331	16.5	8	111	10.9	4	0	1*	140	16.8	54
31-331	17.0	23	104	10.3	10	5	1*	194	21.5	105
31-203	16.9	4	153	13.3	4	4	0	162	17.5	32
32-161	14.2	6	108	8.2	8	0	1	124	12.0	47
32-161	14.4	3	108	8.8	9	0	1*	132	15.7	79
32-161	14.2	6	108	8.6	9	0	3	159	19.3	124
32-161	14.6	3	86	8.8	10	3	3	127	18.6	111

* This amount of bile salt given as whole bile which contains approximately 10 mg. cholesterol.

Zein is an incomplete protein which we have used in a study of bile salt metabolism.   It causes a sharp fall in bile volume and bile salt output and even more conspicuous drop in bile cholesterol.   This deserves further study.

At any rate we see that it is possible to dissociate bile volume, bile salt and bile cholesterol concentration.   In a general way the bile cholesterol-bile salt ratio is about 1 to 100 but this is not constant.

The gist of Table 23 is that egg yolk feeding without bile or bile salt will cause a 40–50 per cent increase of bile cholesterol.   A single egg yolk contains 0.3–0.5 gm. cholesterol.   Bile alone by mouth containing 1 gm. bile salt will cause about the same increase in bile cholesterol.   When larger doses of bile salt (3 gm.) alone are fed we note an increase of over 100 per cent of bile cholesterol and there is no further rise in bile cholesterol if we give this dose of bile salt plus egg yolks.   This point has not been observed by other workers and gives less emphasis to heavy cholesterol feeding (egg yolks).   It is of interest that *blood cholesterol* remains unchanged with bile salt feeding but rises to high levels when bile salt plus egg yolk is fed.   The bile cholesterol elimination remains at the same level in both experiments (Dog 32-161, Table 23).   Under normal physiological conditions with an intact bile circulation and no bile fistula it is probable that heavy cholesterol feeding would cause no reaction (Table 23) or at best a slight rise in bile cholesterol (see Table 24).

Table 24 indicates the *maximum level* to which we have been able to push cholesterol excretion in the bile by means of continued bile feeding plus egg yolk plus large supplementary bile salt additions.   This dog was in perfect physical condition and consumed daily its salmon bread ration.   The supplements added to this ration or given by stomach tube are shown (Table 24).   For 13 days preceding the 1st day given in Table 24, the dog was refed daily the total bile output as collected, minus 10 cc. for routine analysis.   It has been shown elsewhere (21) that refeeding of bile over considerable periods will raise the bile salt output to a level which is sustained at about 7–8 gm. bile salt output per 24 hours.   This dog had not reached this plateau at the time the observations were begun in Table 24 and we note a bile salt output of 4.5 gm. per day.   Meanwhile the bile cholesterol has increased slowly from the control level at the start of bile refeeding—

9.3 mg. to 21.5 mg. When bile salt (3 gm.) is added to the bile re-
feeding we note a great increase in bile cholesterol—42.6 mg. per 24
hours. The peak of cholesterol production follows by 1 day the peak
of bile salt output. Egg yolks added to the bile refeeding increase the

TABLE 24

Bile Cholesterol Influenced by Cholesterol and Bile Salt Feeding
Dog 32-161.

Date	Diet—salmon bread daily	Bile volume daily output	Choles-terol daily output	Bile salt daily output
		cc.	*ms.*	*gm.*
Oct. 10	Bile 178 cc.	198	21.5	4.51
Oct. 11	Bile 198 cc.	218	21.6	4.22
Oct. 12	Bile 220 cc., bile salt 3 gm.	230	16.5	
Oct. 13	Bile 272 cc., bile salt 3 gm.	282	21.0	
Oct. 14	Bile 290 cc., bile salt 3 gm.	300	24.0	
Oct. 15	Bile 286 cc.	308	38.5	8.66
Oct. 16	Bile 300 cc.	332	42.6	6.83
Oct. 17	Bile 246 cc.	256	27.6	
Oct. 18	Bile 228 cc. + 4 egg yolks	238	30.2	
Oct. 19	Bile 302 cc. + 4 egg yolks	312	36.3	
Oct. 20	Bile 255 cc. + 4 egg yolks	265	34.4	
Oct. 21	Bile 301 cc. + 4 egg yolks	332	30.2	6.57
Oct. 22	Bile 253 cc.	273	30.4	5.10
Oct. 23	Bile 234 cc.	244	39.4	
Oct. 24	Bile 218 cc.	228	28.5	
Oct. 25	Bile 262 cc.	272	28.0	
Oct. 26	Bile 248 cc. + bile salt 3 gm. + 4 egg yolks	258	27.2	
Oct. 27	Bile 334 cc. + bile salt 3 gm. + 4 egg yolks	344	34.4	
Oct. 28	Bile 294 cc. + bile salt 3 gm. + 4 egg yolks	304	46.1	
Oct. 29	Bile 364 cc. + bile salt 3 gm. + 4 egg yolks	386	48.3	9.47
Oct. 30	Bile 346 cc.	378	61.0	9.84
Oct. 31	Bile 282 cc.	292	52.2	
Nov. 1	No bile	325	46.8	
Nov. 2	No bile	152	9.1	

bile cholesterol almost as much as does the bile salt but meanwhile the
bile salt output is on the decline.

Maximum figures for bile cholesterol (61 mg. per 24 hours) are ob-
served when we combine egg yolk and bile salt with the whole bile
refeeding. This high level is more than 6 times the base line but

if we consider as normal the output due to bile refeeding then the output is doubled by egg yolk and bile salt supplementary feeding (Table 24).

When bile refeeding is stopped the output falls promptly to the control level on salmon bread diet—9.1 mg. cholesterol per 24 hours. The dog was then fasted for 2 days and the cholesterol fell to 4.3 mg.

Table 25 shows that isatin by mouth or decholin by vein or by mouth will give a definite cholagogue effect without any influence on cholesterol elimination by the bile. In fact as these substances cause some

TABLE 25

Isatin and Decholin Show Cholagogue Effect but Negative Influence on Bile Cholesterol

							Isatin or decholin		
	Control diet				Control diet + isatin or decholin daily				
Dog No.	Duration	Bile volume daily average	Cholesterol average daily output	Bile salt daily average	Duration	Bile volume daily average	Isatin or decholin	Bile salt daily average	Cholesterol average daily output
	days	cc.	mg.	mg.	days	cc.		mg.	mg.
31-331	8	115	8.8		3	155	Isatin—5 gm.		8.0
31-161	10	87	8.3		6	135	Isatin—5 gm.		5.6
31-161	2	90	9.1	1174	2	120	Decholin*	1504	8.4
31-161	3	125	8.8	1100	4	130	Decholin*	1211	3.0
31-161	10	87	8.3	1100	4	238	Decholin—3 gm.	1670	6.6

* Decholin given by vein daily—2 gm.

gastro-intestinal disturbance and occasional vomiting we note more or less decrease in cholesterol elimination. Decholin by vein in one instance caused a good deal of clinical disturbance, very low food consumption and a very low cholesterol output (3.0 mg. per 24 hours). This is practically the fasting level.

It is known that isatin (14) causes no increase in bile salts but decholin does cause a moderate increase in bile salt elimination: This does not compare with the reaction to bile salt by mouth which is subsequently eliminated within 24 hours in amount practically 100 per cent of the intake. We cannot say whether the decholin may be

eliminated as such in the bile as the method used would not detect it. Evidently some of the introduced decholin is linked in the body with taurin to yield taurocholic acid. The cholagogue reaction to decholin is more conspicuous when the drug is given by mouth as compared with intravenous administration. The last two figures for bile salts (1100 mg.) in control periods (Table 25) are general average values.

Table 26 shows a satisfactory experiment in which moderate liver injury was produced by small doses of chloroform by mouth. The

TABLE 26

Bile Cholesterol with Liver Injury and Repair

Dog 32-161.

Date	Diet	Weight	Bile volume daily output	Cholesterol daily output
		kg.	*cc*	*mg.*
Aug. 14	Salmon bread	14.5	90	9.4
Aug. 15	Salmon bread		76	8.1
Aug. 16	Salmon bread		132	8.2
Aug. 17	Salmon bread		108	8.5
Aug. 18	Salmon bread		80	8.2
Aug. 19	Salmon bread		82	7.3
Aug. 20	Salmon bread	14.5	84	9.3
Aug. 21	Salmon bread + chloroform 3 cc.		92	8.2
Aug. 22	Salmon bread + chloroform 3 cc.		50	4.9
Aug. 23	Salmon bread + 100 gm. karo syrup		18	1.6
Aug. 24	Salmon bread + 100 gm. karo syrup	14.6	10	0.4
Aug. 25	Salmon bread + 100 gm. karo syrup		26	1.3
Aug. 26	Salmon bread		42	2.8
Aug. 27	Salmon bread		70	5.4
Aug. 28	Salmon bread	14.5	74	6.0

repair took place promptly and was probably complete in 7–10 days. There was no clinical disturbance, the dog acting normally and eating all food. Bile volume shows a sharp fall to about 10 per cent of normal and the bile cholesterol falls even closer to zero. Bilirubinemia developed with an icterus index of 10 and bile was present in the urine. From published experiments (22) we know that the bile salts in the bile also fell very close to zero. The return toward normal in bile cholesterol is well shown (Table 26) and parallels closely the liver repair and bile salt excretion curve (22) as given elsewhere. From other

experiments we know that in such animals the signs of liver injury are very slight as shown by histological study—a few cells about the central vein showing fat or hyaline necroses. The repair is prompt and completed usually within 7 days.

Table 27 shows a severe liver injury followed by slow return to normal over a period of 4–5 weeks. In connection with other experiments this dog was given hematin intravenously which caused severe and almost fatal poisoning. From autopsy examinations in other dogs we

TABLE 27

Bile Cholesterol with Severe Liver Injury and Slow Repair

Dog 32-161.

Date	Diet	Weight	Bile volume daily output	Cholesterol daily output
		kg.	*cc.*	*mg.*
Feb. 18	Salmon bread	14.3	124	11.5
Feb. 19	Salmon bread		106	11.5
Feb. 20	Salmon bread + 0.2 gm. hematin by vein		110	11.0
Feb. 21	Salmon bread + 0.2 gm. hematin by vein		58	6.5
Feb. 22	Salmon bread + 20 gm. glucose by vein		No bile	—
Feb. 23	Salmon bread		No bile	—
Feb. 24	Salmon bread + * 50 cc. bile by mouth		No bile	—
Feb. 25	Salmon bread + * 50 cc. bile by mouth	13.0	30	—
Feb. 26	Salmon bread + * 50 cc. bile by mouth		44	2.9
Feb. 27	Salmon bread		96	4.6
Feb. 28	Salmon bread	13.6	104	4.7
Mar. 23	Salmon bread	14.2	62	6.1
Apr. 3	Salmon bread	14.0	82	9.3
Apr. 17	Salmon bread	14.0	122	10.3

* This bile contained approximately 5 mg. cholesterol + 0.6 gm. bile salts.

have assurance that there resulted an extensive central liver necrosis, which healed slowly. This dog was severely intoxicated and appeared clinically very sick. Bilirubinemia was severe and the blood fibrinogen fell to 170 mg. per cent. There was bleeding from vein punctures. Clinical improvement began 4 days after the second hematin injection but recovery was slow. There was some loss of weight. For 3 days there was complete suppression of bile flow. The bile cholesterol values came back slowly. In these severe injuries the change of cho-

lesterol output is less spectacular than with slight injuries when the bile flow is not suppressed. These data are in accord with those presented in Table 26.

Blood Destruction and Cholesterol Elimination in Bile

It has been claimed by some investigators and assumed by many others that red cell destruction sets free the cholesterol in the red cell matrix, which logically might well appear in the bile. Other materials coming from red cell destruction (pigments and iron) appear in the liver or bile so why not cholesterol? But experiments indicate that this is not the way of body physiology.

The experiment outlined just below shows no increase in bile cholesterol but rather a slight decrease, probably due to slight intoxication by the hydrazine used to destroy red cells.

Dog 32–161 (see clinical history above). Weight 14 kg., hemoglobin 158 per cent, normal in all respects. The fore period of 10 days showed a somewhat low normal cholesterol daily output of 6.3 mg. During a 4 day period the dog was given subcutaneously 100 mg. daily of acetylphenylhydrazine. This caused a drop in the hemoglobin level to 86 per cent. Calculating the destroyed hemoglobin on the basis of the dog's weight and our general experience with anemia in dogs, it is safe to say that not less than 100 gm. hemoglobin were destroyed. If any cholesterol is to be derived from hemoglobin destruction and appear in the bile, this would seem an adequate test. During the 4-day period of hydrazine administration and the subsequent 10 days, the bile cholesterol averaged 5.5 mg. per day. The after period of 16 days shows a bile cholesterol daily output of 7.6 mg. At the end of this last period the hemoglobin level had come back to 112 per cent. The dog was fed the standard control salmon bread diet throughout and the weight was unchanged.

DISCUSSION

Possibly clinical treatment of abnormalities of the biliary system has not taken into consideration some of the facts established by experimental study of the bile. This may not be the place for a discussion of clinical problems but it may be proper to indicate that certain cholagogues can be used with advantage in human cases presenting irritation or inflammation of the biliary tree. Under these conditions it is recognized that stasis of bile and high cholesterol concentration may favor the precipitation of cholesterol with subsequent building

up of gall stones. It is logical to assume that on such occasions an active flushing of the biliary ducts by means of some cholagogue might forestall the unfortunate precipitation of debris and cholesterol. Also bile salts in addition to their active cholagogue effect will appear in the bile and help to hold any excess of cholesterol in solution. It is even conceivable that a small soft precipitate of cholesterol under such conditions might go back into solution, as bile salts effect rapid solution of cholesterol. In the dog's gall bladder it has been shown (1, 7) that human gall stones will be dissolved during the course of many weeks.

The cholesterol-bile salt ratio is about 1 to 100 in the bile fistula bile but considerable variations may be noted. The ratio in the blood must be vastly different although we cannot say how much bile salt is to be found in the circulating blood. As the normal blood plasma contains about 200 mg. cholesterol per 100 cc., if the same ratio obtained we should find about 20 gm. bile salt per 100 cc. plasma which is ridiculous. It is probable that the blood plasma contains only a few milligrams of bile salt per 100 cc. but present methods do not permit us to measure this with any accuracy.

Therefore we have a considerable amount of cholesterol in circulation—for example a 10 kg. dog would have a plasma volume of 500 cc. and a cholesterol concentration of 150 to 300 mg. per cent—or 750 to 1500 mg. in circulation. From this reservoir of ± 1 gm. plasma cholesterol we have only a trickle of 10–20 mg. per day appearing in the bile. Meanwhile the feeding of cholesterol and bile salt may change the level of the plasma reservoir of cholesterol by large amounts. All this would point to the bile cholesterol elimination as a minor shunt for certain surplus material. We do not accept this conclusion without protest and believe that the bile cholesterol is related in some way to the important internal cholesterol metabolism which goes on in the liver cell.

Because cholesterol and bile salt have marked similarities in their structural formulas—both contain the same four ring nucleus—it has been claimed that cholesterol is the precursor of bile salt. This thesis has been shown by Smith and Whipple (13) to be untenable. But may a surplus of bile salt be changed to cholesterol? This seems to be unlikely on theoretical grounds and there is no real support for this

hypothesis on experimental grounds. The body seems able to dispose of any surplus of bile salts without any demonstrable increase in cholesterol stores or elimination. However the experimental data are not adequate as yet to exclude this possibility.

SUMMARY

Under uniform diet conditions the normal bile fistula dog will eliminate pretty constant amounts of cholesterol—about 0.5 to 1.0 mg. cholesterol per kilo per 24 hours.

Diets rich in cholesterol (egg yolk) will raise the cholesterol output in the bile but compared to the diet intake (1.5 gm. cholesterol) the output increase in the bile is trivial (5–15 mg.). Calves' brains in the diet are inert.

Bile salt alone will raise the cholesterol output in the bile as much and often more than a cholesterol rich diet.

Bile salt plus egg yolk plus whole bile give maximal output figures for bile cholesterol—60 mg. per 24 hours.

Liver injury (chloroform) decreases both bile salt and cholesterol elimination in the bile.

Blood destruction (hydrazine) fails to increase the bile cholesterol output and this eliminates the red cell stroma as an important contributing factor.

Certain cholagogues (isatin and decholin) will increase the bile flow but cause no change in cholesterol elimination.

The ratio of cholesterol to bile salt in the bile normally is about 1 to 100 but the bile salts are more labile in their fluctuations.

The ratio is about reversed in the circulating blood plasma where the cholesterol is high (150–300 mg. per cent) and the bile salt concentration very low.

Cholesterol runs so closely parallel to bile salt in the bile that one may feel confident of a physical relationship. In addition there is a suspicion that the bile cholesterol is in some obscure fashion linked with the physiological activity of hepatic epithelium.

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III. BLOOD PLASMA CHOLESTEROL

FLUCTUATIONS DUE TO LIVER INJURY AND BILE DUCT OBSTRUCTION

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As the fluctuations of the bile cholesterol were followed in bile fistula dogs, Paper II, changes were observed which suggested that a study of *blood plasma cholesterol* might yield information of value. Under varying conditions the fluctuations in bile and blood plasma cholesterol might be correlated and lead to a better understanding of cholesterol metabolism. Much work has been done to indicate the changes in blood plasma cholesterol which might be of significance in clinical diagnosis of human disease. This is referred to below in a brief review of this literature.

It is notorious that human disease presents a very complex mixture of abnormal functions, and consequently it is at times extremely difficult to evaluate data derived from study of human material. Little experimental work has been done with animals to produce one single type of injury or abnormal condition and observe the alterations in blood plasma cholesterol which might follow. From this study one is forced to conclude that like many other tests for liver function or liver disease the blood plasma cholesterol may give information of some value but diseased conditions may be present without significant disturbance of total blood cholesterol or of the esterified cholesterol ratio.

A drop in the ratio of cholesterol esters to total cholesterol of the blood plasma was first observed by Feigl (7) in cases of acute yellow atrophy. This phenomenon was more extensively investigated by Thannhauser and Schaber (15) who explained this change on the basis of injury of the liver with consequent impairment of function of an enzyme of the liver cells which is effective in hydrolyzing esterified cholesterol. Thannhauser (14) has demonstrated this enzyme and its action in bile. Gardner and Gainsborough (8) interpreted the low cholesterol ester values in pathological states of the liver as being due to failure of absorption of cholesterol

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as a result of lack of bile in the intestine, or as an alternative hypothesis, that in the absence of fat intake from the intestine, the body utilizes the fatty acid already combined with cholesterol—a de-esterification.   Epstein (6) has noted hypocholesteremia and dissociation of the normal ratio of esterified to total cholesterol in patients with parenchymatous liver disease.   He has also commented on hypercholesteremia occurring in certain cases of biliary obstruction.   Mjassnikow (12) reports hypocholesteremia in liver injury caused by phosphorus and arsphenamine. His experimental animals were dogs and rabbits.

Experimental Methods

We have used the colorimetric method of Bloor (1) and Bloor and Knudson (3) for the determination of blood plasma cholesterol, with minor modifications described in Paper I.   It is worth while to note here again that in a long series of comparisons of the colorimetric and digitonin methods for the determination of blood plasma cholesterol, Bloor (2) has found the colorimetric method to run consistently about 20 per cent higher than the digitonin method, and expresses the opinion that the colorimetric value more closely approximates the actual.   This is in accord with observations made by one of us (Paper I).

The dogs are bled every morning at the same time and fed in the early afternoon each day, so that the blood samples are free from the questionable influence (9, 4) of alimentary absorption.   Approximately 10 cc. of blood drawn from the jugular vein is received into a 15 cc. calibrated hematocrit tube containing 2 cc. of a solution of 1.4 per cent sodium oxalate and centrifugalized for 35 minutes at a speed of 2600 R.P.M.   The same hematocrit tube is used for the same animal each day.

Determinations of the icterus index are made by the method described by Cutten *et al.* (5) and the values recorded are in milligrams of bilirubin per liter of plasma. The method of Jones and Smith (10) is used in the determination of fibrinogen.

Parenchymatous liver injury is produced by giving small doses of chloroform by mouth.   The chloroform is suspended in a slightly viscous solution of starch or dissolved in small quantities of cotton seed oil and given by stomach tube. The chloroform dissolved in cotton seed oil is better tolerated.

EXPERIMENTAL OBSERVATIONS

The effect of parenchymatous liver injury upon the blood plasma cholesterol was studied in ten experiments performed on nine dogs, in some as uncomplicated parenchymatous injury and in others associated with biliary obstruction.   The results of the simple parenchymatous injury experiments are uniform (see Chart A).   After an adequate control period the animals are given small daily doses of chloroform by mouth in a starch solution or in cotton seed oil.   There results a progressively increasing jaundice and after varying lengths

)f time the blood plasma cholesterol values begin to drop. At the)eak of liver injury, as indicated by marked jaundice and intoxication, the ratio of esterified cholesterol to the total cholesterol decreases to 30 per cent or less (normal ratio 40–70 per cent). If the dogs are allowed to recover, the ester ratio and the values for free and esterified cholesterol mount rapidly with subsidence of the jaundice. Sometimes recovery was attended by slightly higher plasma cholesterol values than noted in the control periods.

TABLE 31

Blood Plasma Cholesterol—Chronic Chloroform Liver Injury

Dog 32-143.

Date	Weight	Food consumed	Icterus index	CHCl₃ by mouth daily	Total cholesterol	Esters, cholesterol	Ester ratio
	kg.	*per cent*		*cc.*	*mg. per cent*	*mg. per cent*	*per cent*
Mar. 26–Mar. 30	20.7	100	0	0	155	89	58
Mar. 31–Apr. 8	21.3	100	14	4	163	88	54
Apr. 9–Apr. 17	21.0	100	25	4	145	81	55
Apr. 18–Apr. 26	19.5	100	25	4	119	59	48
Apr. 27–May 5	19.5	100	32	9	147	75	51
May 6–May 17	18.8	80	23	15	131	64	48
May 18–May 23	17.6	50	38	15	124	60	48
May 24		25	53	20	131	49	37
May 25		50	58	20	106	48	45
May 26		14	48	20	96	32	33
May 27	17.6	0	50	20	89	25	28
May 28		0	62	20	89	30	34
May 29		0	40	20	106	27	25
May 30		0	60	20	120	37	31
May 31		0	60	20	190	28	15

In all cases when the ratio of esterified cholesterol dropped to 30 per cent or less of the total cholesterol the animals were critically ill. The ratio of esterified cholesterol taken just before death varied from 0 per cent to 26 per cent of the total.

Table 31 (Dog 32-143) shows the effect upon the blood plasma cholesterol of parenchymatous liver injury caused by repeated daily doses of chloroform by mouth.

Previous to this experiment this dog had been subjected to 1 hour of chloroform anesthesia (see Table 32) and to a course of chloroform by mouth with development of moderately severe parenchymatous injury.

A most interesting finding in this experiment was the large amounts of chloroform which were tolerated over a long period of time. Over a 2-month period this animal was given over 12 times the amount of chloroform necessary to injure severely the liver of a normal animal over a 2-week period. MacNider (11) has reported that liver cells may acquire resistance to chloroform and uranium after previous injury of the liver by these poisons.
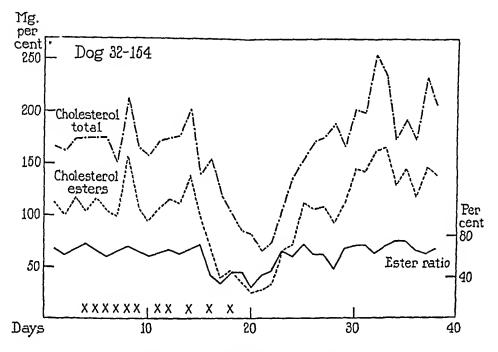
CHART A. Blood plasma cholesterol—chloroform liver injury.
X = 3 cc. chloroform by mouth.

In the table the first seven horizontal lines indicate average values for the periods noted. In the period from Apr. 18 to Apr. 26 the dog was fasted for 5 consecutive days during which the chloroform was continued. In spite of fasting the ratio of esterified to total cholesterol remained normal, 48 per cent.

For 50 days the dog was given chloroform by mouth in amounts as indicated, and during this period there was persistent jaundice but no significant change in the blood plasma cholesterol. In the last 8 days of the experiment the jaundice became more marked. The animal showed definite changes in the blood plasma cholesterol, with rapid physical decline, and drop in the ratio of esterified cholesterol to 15 per cent of the total on the day of death. During the latter part of the experiment the animal left food and in the last 5 days did not eat.

Autopsy revealed generalized jaundice. The extra-hepatic biliary ducts were patent and there was bile in the duodenum. The liver, grossly, was firm, uniformly yellow in color with no alteration of the lobulation. Histologically the liver showed severe fatty degeneration with normal appearing liver cells only in the portal regions. There was also a terminal pneumonia. Other organs were normal.

Chart A, Dog 32-154, illustrates a similar experiment in which the dog developed pronounced liver injury within a period of 16 days. Eleven doses of chloroform 3 cc. were given. On the 15th day of the experiment there was significant change in the blood plasma cholesterol. As hypocholesteremia developed, the ratio of esterified to total cholesterol began to drop and continued to fall until on the 20th day it was 30 per cent of the total. At this point the dog was severely intoxicated and jaundice was marked. Sugar was given by vein during the day with consequent betterment of the animal's condition. Recovery as indicated by rise in the blood plasma cholesterol and decrease of bilirubinemia was rapid. The ratio of esterified to total cholesterol returns to normal before the control levels of total cholesterol values are reached.

Two more dogs, 31-169 and 32-172, gave similar results under the same experimental conditions. Dog 31-169 died at the height of liver injury and autopsy showed generalized jaundice and a fatty liver. There was a duodenal ulcer. Histologically in the liver there was fatty degeneration of practically all liver cells.

The results of acute liver damage caused by 1 hour of chloroform anesthesia are given in Table 32, Dog 32-143. In the days following anesthesia the dog was intoxicated and showed marked bilirubinemia. Surprisingly enough in the face of the results recorded above the blood plasma cholesterol did not vary beyond the usual diurnal range.

Dog 32-265 was fasted for 48 hours before chloroform anesthesia of 1 hour duration. Following this, jaundice was marked but again blood plasma cholesterol values remained normal. The dog was killed under ether anesthesia 48 hours after the chloroform was administered. Autopsy showed moderate generalized icterus. There was definite diminution of fibrinogen. The liver showed grossly central necrosis and histologically there was hyaline necrosis involving about half of the liver lobule with a peripheral zone of fatty cells. In the portal regions normal appearing liver cells persisted.

The effect of *biliary obstruction* upon the blood plasma cholesterol was studied on six dogs. Four of these animals were obstructed surgically after a period of control observation, the others were bile fistula

dogs of the type developed by Rous and McMaster (13) which had previously been studied in connection with bile and blood plasma cholesterol prior to obstruction. All of these animals were completely obstructed as evidenced by absence of bile pigments in the feces, and marked cholemia. At autopsy careful investigation of the biliary tract was made to check the completeness of biliary obstruction.

TABLE 32

Blood Plasma Cholesterol—Acute Liver Injury by Chloroform
Dog 32-143.

Date	Weight	Food consumed	Jaundice plasma	Total cholesterol	Esters, cholesterol	Ester ratio
	kg.	*per cent*		*mg. per cent*	*mg. per cent*	*per cent*
Dec. 15	21.0	100	0	96	64	67
Dec. 16		100	0	106	67	62
Dec. 17		100	0	99	62	64
Dec. 18	20.5	100	0	104	67	64
Dec. 19				0	102	65	64

1 hr. CHCL₃ anesthesia

Date	Weight	Food consumed	Jaundice plasma	Total cholesterol	Esters, cholesterol	Ester ratio
Dec. 20	21.0	100	+++	111	65	59
Dec. 21		100	+++	111	58	55
Dec. 22		100	++	130	78	60
Dec. 23	21.0	100	+	121	76	63
Dec. 24		100	+	93	44	47
Dec. 25		100	+	102	58	57
Dec. 26	20.0	100	Trace	119	62	52
Dec. 27		100	Trace	102	54	53

The effect on blood plasma cholesterol resulting from bile duct obstruction is shown in Table 33, Dog 32-326. The first four lines give average figures for the periods indicated by the dates.

After a control period of a week, the common bile duct was ligated and cut under ether anesthesia. Jaundice developed promptly and the dog began to leave food with resulting slight decrease in weight. Blood plasma cholesterol levels were followed for 27 days. During this period there was no significant change in the ratio of esterified to total cholesterol. The amount of both total and esterified cholesterol, however, did increase definitely above the control levels. Chloroform by mouth was started and immediately there was a rapid decrease in the total blood plasma cholesterol with even more marked drop in the esterified cholesterol.

Consequently the ester ratio falls below normal and remains low. On the last 2 days of life there were no cholesterol esters demonstrable in the blood. 5 days before death when the ester ratio was at 14 per cent the fibrinogen was 109 mg. per cent, indicating very definite liver injury.

Autopsy revealed generalized jaundice with complete obstruction of the common bile duct. The gall bladder and biliary ducts were dilated and filled with thick

TABLE 33

Bile Duct Obstruction with Superimposed Chloroform Injury

Dog 32-326.

Days	Weight	Food consumed	Icterus index	CHCL₃ by mouth	Total cholesterol	Esters, cholesterol	Ester ratio
	kg.	*per cent*		*cc.*	*mg. per cent*	*mg. per cent*	*per cent*
Sept. 12–Sept. 18	8.4	100	0	0	172	76	45
Common bile duct ligated and cut							
Sept. 19–Sept. 25	8.6	50	20	0	211	99	49
Sept. 26–Oct. 2	7.5	50	20	0	187	88	46
Oct. 3–Oct. 12	7.3	50	23	0	206	103	51
Oct. 13	7.2	50	23	0	255	127	50
Oct. 14		20		0	335	121	36
Oct. 15		50	21	0	338	125	37
Oct. 16	7.1	50		0	301	165	55
Oct. 17		47	24	4	232	151	65
Oct. 18		30		4	221	73	33
Oct. 19		20	26	4	83	27	33
Oct. 20	7.2	32		0	68	27	39
Oct. 21		20	27	4	53	19	36
Oct. 22		40		0	36	12	34
Oct. 23		6	30	4*	87	12	14
Oct. 24	6.6	0		4	54	14	26
Oct. 25		0	31	8	40	13	33
Oct. 26		0		8	45	12	26
Oct. 27	6.1	0	46	8	45	0	0
Oct. 28				8	46	0	0

* Fibrinogen 109 mg. per cent on this date.

dark green bile. The liver was bile stained and very fatty. No gross evidence of infection was seen. There was a duodenal ulcer. The other organs appeared normal. Histologically the liver showed central fatty degeneration with normal appearing liver cells in the portal regions. Bile canaliculi were distended with brown colloid material. Phagocytic cells in the liver sinusoids contain brown pigment granules.

Table 34, Dog 32-341, shows the results of long continued biliary obstruction with subsequent superimposed chloroform liver injury. Again average figures are given for several periods as indicated by the dates.

This dog was studied for 41 days after obstruction before chloroform was given. During the simple obstructive period there was definite increase in the total and

TABLE 34

Bile Duct Obstruction with Superimposed Chloroform Injury
Dog 32-341.

Date	Weight	Food con- sumed	Icterus index	CHCL₃ by mouth	Fibrino- gen	Total choles- terol	Esters, choles- terol	Ester ratio
	kg.	per cent		cc.	mg.	mg. per cent	mg. per cent	per cent
Sept. 12–Sept. 18	13.3	100	0			270	143	52
Common bile duct ligated and cut								
Sept. 19–Sept. 25	15.9	80	10			289	144	50
Sept. 26–Oct. 2	13.8	75	30			388	204	56
Oct. 3–Oct. 9	13.6	70	36			361	180	49
Oct. 10–Oct. 16	12.6	50	42			279	137	49
Oct. 17–Oct. 23	12.4	50	50			381	185	48
Oct. 24–Oct. 30	12.4	100	30			350	193	52
Oct. 31–Nov. 6	12.3	20	52	6		342	170	50
Nov. 7–Nov. 13	11.6	20	54	8	138	111	28	25
Nov. 14	11.0	16	54	10	167	108	23	21
Nov. 15	10.9	18		10		152	27	18
Nov. 16		16	56	20	195	140	25	18
Nov. 17		0				121	25	21
Nov. 18	10.8	10	51		204	110	24	22
Nov. 19		20				164	33	20
Nov. 20	10.5	0	45		306	132	26	20
Nov. 21		0				173	40	23

esterified blood plasma cholesterol above the control levels. The ratio of esters to total cholesterol remains normal. A week after daily administration of chloroform (6 cc.) by mouth the values for both total and esterified cholesterol decrease markedly. The ester ratio drops to 25 per cent of the total as compared with normal of 50 per cent. These lower levels are maintained throughout the rest of the experiment. Food consumption was poor, particularly after chloroform administration was commenced. On Nov. 16 after 10 days of severe liver injury an attempt was made to bring back the dog to normal by administration of intrave-

nous glucose. Apparently the margin of hepatic safety had been passed as the animal died after 4 days of such treatment. Following sugar therapy the fibrinogen levels rose to normal while there was no change in the cholesterol level of the plasma. Before glucose in saline was given on Nov. 16, the dog's red cell hematocrit was at 40 per cent; following glucose this dropped to 20 per cent at which level it remained until death, which occurred despite transfusion. In face of this obviously great dilution of the total plasma volume, there was no drop in the total cholesterol.

TABLE 35

Chronic Bile Duct Obstruction with Superimposed Cholangitis

Dog 31-203.

Date	Weight	Food consumed	Icterus index	Total cholesterol	Esters, cholesterol	Ester ratio
	kg.	*per cent*		*mg. per cent*	*mg. per cent*	*per cent*
July 14	15.7	100	30	315	177	56
July 15		100	30	291	119	41
July 16		100	32	376	184	49
July 17	15.6	100	28	327	164	50
July 18		100	30	322	183	57

Interval of 52 days.   Animal now progressively ill

Date	Weight	Food consumed	Icterus index	Total cholesterol	Esters, cholesterol	Ester ratio
Sept. 6	15.9	100	28	95	33	35
Sept. 7		100	25	63	28	44
Sept. 8		100	28	81	27	33
Sept. 9		0	35	83	20	24
Sept. 10		100		106	28	26
Sept. 11		100	55	107	24	22
Sept. 12		0	40	57	13	23
Sept. 13		0	45	52	14	26
Sept. 14		0	40	74	13	17
Sept. 15	14.3	0	48	71	13	16

Autopsy showed generalized jaundice, completely obstructed dilated bile ducts filled with dark green bile. The liver was fatty and histologically showed generalized fatty degeneration with scattered liver cells undergoing hyaline necrosis, with resulting polymorphonuclear leucocytic infiltration. Two duodenal ulcers were present. The other organs appeared normal.

Two other experiments of the same type on Dogs 32-270 and 32-380 gave similar results.

Table 35, Dog 31-203, presents some interesting data obtained from study of a chronically obstructed bile fistula dog which had been used

for 18 months on bile salt study and had been totally deprived of bile with the exception of an occasional short period. 12 days after total obstruction occurred, a study of plasma cholesterol was begun.

The table shows a high level for both total cholesterol and esters but a normal ester ratio. For 52 days the dog ate all of its food and continued active, but at the end of this period began to leave food, became inactive and appeared to be going down hill. Cholesterol studies at this time showed a very marked change. Totals of cholesterol are low and the ratio of esters to the total is much below the normal with progressive drop until the level of 16 per cent is reached.

During these last 10 days the dog had three large gastro-intestinal hemorrhages. The red cell hematocrit dropped from 34 to 10 per cent. The animal was very weak and would not eat. Transfusions did not raise the hemoglobin. On the 10th day a large fresh blood clot was vomited. The dog was killed under gas anesthesia, and autopsy performed immediately. The essential findings were generalized jaundice, multiple infected infarcts of varying sizes in the spleen and kidneys. Throughout the gastro-intestinal tract were many fresh and old blood clots. In the duodenum just beyond the pylorus there were two sharply and deeply punched-out ulcers with a central point from which the hemorrhages were occurring.

The liver was studded with abscesses, measuring from 1 cm. to 4 cm. in diameter. The intra-hepatic ducts were distended with pus as were the extra-hepatic and common bile ducts. The ducts had thick walls. The fistula cannula was in place but plugged with inspissated bile. No communication was found between the bile duct and the duodenum. Histologically, the liver showed subacute cholangitis with abscess formation.

Another bile fistula dog (31-331) became totally obstructed and was kept for 7 months on a diet of white bread, klim and water with no bile in the intestinal tract. This animal showed high normal blood plasma cholesterol values with the normal ratio of esters to total cholesterol. These bile fistula animals also maintain a normal cholesterol ester ratio in their blood plasma all through the period in which the fistula is draining and this despite total absence of bile from the intestine.

DISCUSSION

From a consideration of these results it is evident that hypocholesteremia with dissociation of the ratio of esterified to total cholesterol is not due simply to parenchymatous liver injury. In marked acute chloroform liver injury normal values for cholesterol are found whereas

hypocholesteremia occurs in chronic chloroform injury or in injury acutely produced by this drug or infection in dogs with long continued biliary obstruction. One can safely state that the above mentioned changes in blood plasma cholesterol are *related* to chronic severe injury of the liver. This suggests the possibility that these alterations in the metabolism of plasma cholesterol are a secondary manifestation of a chronic derangement of hepatic function.

In all the obstructed dogs we have found a moderate increase in the free and combined cholesterol, but the icterus index has increased from zero to 50 meanwhile. Therefore the increase in cholesterol does not parallel the degree of jaundice as other investigators have maintained. In Paper II it has been shown that in dog's bile there are eliminated about 20 mg. of free cholesterol daily. If, as has been claimed, hypercholesteremia is a result of biliary obstruction alone, then the free cholesterol in the blood should increase much more than the esterified cholesterol, and so dissociation of the ratio of esterified to total cholesterol would be expected. In our dogs a normal ester ratio was maintained during long periods of total obstruction.

The hypercholesteremia observed in biliary obstruction may not necessarily be due to changes within the liver itself. In diabetes and nephrosis high blood cholesterol values are found but this is not attributed directly to changes in the epithelium of the pancreas and kidney.

Quantitative study of blood cholesterol and cholesterol esters has been proposed as a simple test to differentiate between obstructive lesions and parenchymatous lesions of liver. In simple obstruction, the total cholesterol may be elevated with the esters rising in proportion or increasing relatively whereas in parenchymatous injury the total cholesterol will decrease and the combined cholesterol drop even to the point of disappearance in severe injuries. This is true in a great many instances but one must bear in mind the results in acute chloroform poisoning where it has been shown that acute injury does not necessarily change the blood cholesterol.

It should be stressed that in a dog with chronic biliary obstruction with total blood cholesterol values of twice normal (Table 33) this hypercholesteremia may be promptly reduced below normal by chloroform poisoning. Therefore while biliary obstruction may cause high

values for blood cholesterol, the combination of liver injury (chloroform) or biliary infection (Table 35) will cause a prompt fall to subnormal levels. This indicates again the futility of the diagnostic index that hypercholesteremia means bile duct obstruction and hypocholesteremia with dissociation of the ratio of esterified to total cholesterol means liver parenchyma injury.

It is important to emphasize the significance of the ratio of esterified to total cholesterol as a criterion of impairment of liver function over any change that may occur in the total plasma cholesterol. The normal maximum variation which may occur in the total plasma cholesterol is very wide, but the ratio of esterified to total plasma cholesterol is more constant (from 40–70 per cent). The constancy with which this ratio is maintained in dietary extremes and disturbed liver function indicates a physiological process capable of great compensatory effort. When values for the ratio of cholesterol esters of the plasma fall below the "low normal" it is an indication of impairment of the functional capacity of the liver.

It is obvious that the cholesterol analysis like other tests for liver function and liver injury has its limitations and alone will not write the diagnosis for the clinician. The test brings evidence which has weight but must be considered together with all other available data to give a better understanding of liver function and disease.

SUMMARY

Hypocholesteremia with dissociation of the normal ratio of esterified to total cholesterol is related to chronic liver injury caused by chloroform.

Hypercholesteremia may develop after prolonged biliary obstruction.

The hypercholesteremia of chronic biliary obstruction may be promptly reduced below normal by chloroform poisoning or bile duct infection.

Acute injury of liver due to chloroform anesthesia may cause no change in blood plasma cholesterol.

Absence of bile in the intestine with faulty fat absorption does not cause the development of hypocholesteremia with dissociation of the ester ratio.

Poor food consumption or short periods of fasting may cause no change in blood plasma cholesterol.

Liver cells injured by chloroform may subsequently become resistant to chloroform.

After prolonged biliary obstruction, the liver is apparently more sensitive to small doses of chloroform by mouth.

Analysis of blood plasma cholesterol may have a clinical application in differentiation between simple obstructive and parenchymatous lesions of the liver.

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A SEROLOGICAL DIFFERENTIATION OF SPECIFIC TYPES OF BOVINE HEMOLYTIC STREPTOCOCCI (GROUP B)

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In a preceding paper (1) it was shown that hemolytic streptococci isolated from man, lower animals, and dairy sources can be differentiated into serological groups, which correspond, in general, to those described by other investigators on the basis of cultural and biochemical characteristics. The experimental evidence indicates that the so called C substances, upon which the group specificity depends, belong to the general class of carbohydrates, and that in each group this determinative substance is chemically distinct and serologically specific. In the particular series of strains on which this investigation was based the serological groups were found to bear a certain relationship to the origin of the cultures: Group A is composed largely of strains of human origin; Group B of those derived from mastitis in cows and from normal milk; Group C of those from various lower animals, including a number from cattle; Group D comprises in this series hemolytic streptococci from cheese; and Group E is made up of a few strains isolated from certified milk. Probably additional groups exist.

In still earlier work (2) the occurrence of serological types within Group A was determined by means of agglutination reactions combined with mouse protection tests. Later (3) it was demonstrated that specific types differentiated among Group A strains by use of the precipitin reaction correspond with those previously classified by the methods of agglutination and mouse protection. In these later studies, the so called M substances, responsible for type specificity among the members of Group A, were shown to be protein in nature. This is in contrast to the fact that the so called S substances, which determine the type specificity of certain encapsulated bacteria, have usually proved to be polysaccharides. With the possibility in mind that type specificity among certain hemolytic streptococci might in some instances be dependent upon polysaccharides, a search for mucoid and encapsulated strains was undertaken. No human strains, however, were encountered containing any other type-specific substance than the protein, M. It was soon found, on the other hand, that the members of Group B, chiefly of bovine origin, fell into distinct types, the serological specificity of which was, indeed, determined by the presence of specific polysaccharides.

The purpose of the present paper is to define the serological types found in Group B hemolytic streptococci, those derived from mastitis

in cows and from normal milk, and to present the data thus far available with respect to the chemical composition and immunological properties of the type-specific polysaccharides of two of the three specific types differentiated within this group. For purposes of comparison the data are also included showing the chemical properties of the carbohydrate, C, the group specific substance common to all organisms of Group B irrespective of their type differentiation.

Differentiation of Specific Types of Group B Hemolytic Streptococci by the Method of the Precipitin Reaction

Twenty-one strains, classified serologically as members of Group B by means of the precipitin reaction, were available for the present study. Their origin and group characteristics—cultural, biochemical, and serological—have been recorded previously (1). While the existence of specific types within this group was readily apparent, the ultimate differentiation was greatly facilitated by the production and use of antisera in which the type-specific precipitins were the dominant antibodies. The following methods, while by no means infallible, were of assistance in accomplishing this classification.

Methods

1. Immunization of Rabbits.—The most satisfactory method for inducing antibodies against polysaccharides was that of intravenous injection of rabbits with formalinized cultures. The cultures were prepared by resuspending the bacterial cells from 16 hour broth cultures in 0.85 per cent NaCl solution containing 0.2 per cent formalin. The volume was 1/20 that of the original culture. After 48 hours in the ice box, the bacterial suspensions were usually sterile. Immediately before use, these suspensions were diluted 20 times with physiological salt solution; then repeated courses of 1 cc. amounts were injected intravenously into rabbits daily for a week, followed by a week's rest. The antibody response depended somewhat upon the total dosage and the number of series of injections given. Thus, after one series of injections, the precipitin for the group-specific polysaccharide, C, was often present almost to the exclusion of other antibodies. After the second or third series of injections with the same dosage as the first, the concentration of the group-specific anti-C precipitin increased and the type-specific antibody began to appear. Thereafter, the anti-C precipitins frequently decreased in concentration or even disappeared completely, while the type-specific anti-S precipitins usually increased. Sometimes, however, one or the other of these antibodies was markedly predominant in the serum throughout the course of the immunization, while in other instances the titer of both antibodies might be

equally high or low. In some of the rabbits which showed a poor antibody response, the dosage was increased after four or five courses of injections to as much as 20 times the original amount. After this prolonged and intensive immunization, some rabbits finally developed precipitins in their sera for extracts of the homologous organism; but these antibodies proved to be not entirely type-specific, although they tended to show a certain degree of strain specificity. They were, moreover, antibodies for protein substances rather than for polysaccharides. The precipitin tests with this kind of antiserum were confusing, since the predominant reaction appeared to indicate a strain specificity dependent upon a protein. The exact immunological relationship of this protein to the other protein constituents of the cell could not be determined, even though numerous absorption experiments were performed for the purpose. For type differentiation, therefore, it was found desirable not to resort to large doses of formalinized culture in the immunization of rabbits but to use small doses in repeated series of injections. In every case it was essential to determine as far as possible, the kinds and relative concentrations of antibodies present in a given serum before employing it for classification of hemolytic streptococci.

2. Specific Absorption of Antisera.—In the experiments here reported it was possible to use the direct precipitin test for differentiating the hemolytic streptococci of Group B into specific types. This was accomplished by employing sera containing a high titer of anti-S precipitins and low titers of anti-C precipitins and antiprotein precipitins of undetermined specificity. When purely type-specific antisera were lacking, however, the following method was employed. Sera containing a mixture of antibodies of relatively high titers were absorbed with heat-killed streptococci belonging to Group B but heterologous in type to the strain used in preparing the serum. Control serum was absorbed with the homologous strain. The bacteria, centrifuged from broth cultures, were suspended in physiological salt solution and heated at 56°C. for 1 hour. The centrifuged organisms were mixed with an equal volume of undiluted serum. After 30 minutes' incubation at 37°C., the serum was separated from the bacteria by centrifugation and tested for heterologous precipitins. If absorption was incomplete, the process was repeated. It was thus possible to study the antibody content of sera and to determine certain relationships between individual strains.

3. Preparation of Extracts.—The extracts were prepared by heating the bacteria suspended in N/20 HCl as previously described (1). This crude extract was used in some instances, but in others, further separation and concentration of the active substances was accomplished by fractional precipitation with alcohol. After the addition of two to three volumes of 95 per cent ethyl alcohol, a precipitate appeared which was separated and, on resolution, was found to contain most of the type-specific S substance, together with more or less irrelevant protein material. The confusion arising in the serological relationships of these proteins was avoided as much as possible by using antisera with as little antiprotein precipitin as possible.

The supernatant alcoholic fluid, which was removed from the precipitated S substance, was concentrated on the steam bath and was found to contain most of the group-specific carbohydrate, C.

4. Digestion with Trypsin.—To test the effect of trypsin on the serologically active substances extracted from the bacteria, the following technique was employed. A 2 per cent suspension of Fairchild's trypsin in physiological salt solution was incubated for 10 minutes in a water bath at 37°C. After centrifugation, the supernatant fluid was made alkaline to phenolphthalein. Part of the enzyme solution was inactivated by heating for 10 minutes in a boiling water bath. Equal parts of the active and inactive solutions were mixed respectively with equal parts of the bacterial extract. If necessary, toluene and chloroform were added as preservatives. The mixtures were incubated for periods varying from 10 minutes to 6 days. Samples were removed at intervals and the trypsin inactivated by heating for 10 minutes in a boiling water bath. Precipitin tests were made on these samples to determine whether the serologically active substances originally present in the extracts were undigested. The activity of the trypsin was insured by proving its ability to digest a known type-specific protein, M, of a Group A strain (4). Fresh trypsin was added if shown necessary, and the digestion was continued as long as desired.

5. The Precipitin Test.—To serial dilutions of the extract, a constant volume of 0.2 cc. of undiluted serum was added and layered. Usually, tubes were set up containing 0.4 cc., 0.1 cc., and 0.025 cc. of extract in a volume of 0.4 cc. Controls with physiological salt solution plus serum, and with extract plus normal serum were included. After 20 minutes in a water bath at 37°C., the mixtures were observed for ring formation, then shaken and reincubated. Preliminary readings were made after 2 hours in the water bath, and final readings after standing overnight in the ice box.

The results of the precipitin reactions with crude hydrochloric acid extracts are given in Table I. The data recorded in the last three columns show that all 21 strains are members of Group B, as indicated by their positive reactions with an antiserum in which the group, or anti-C, precipitin, is predominant. The results recorded in Columns 2 to 10 show the serologically specific types into which these organisms have been subdivided by the use of antisera containing the type-specific, or anti-S, precipitin as the dominant immune body. Using unabsorbed antisera, all except three of the 21 strains of hemolytic streptococci studied were differentiated serologically into three sharply defined and specific types: four strains in Type I, eight in Type II, and six in Type III. Extracts of most strains of a given type reacted specifically with an antiserum of the homologous type, and gave little or no cross-reaction with immune sera of the heterologous types. Usually, in fact, no reaction was observed in heterologous sera at the end of the incubation period, and evidence of slight cross-precipitation

Precipitin Reactions of Group ...

Extract from	Specific type antisera (anti-S), unabsorbed									Group B antiserum (anti-C), 0.2 cc		
	Type I, 0.2 cc			Type II, 0.2 cc			Type III, 0.2 cc					
	0.4	0.1	0.025	0.4	0.1	0.025	0.4	0.1	0.025	0.4	0.1	0.025
Type I strains												
(1) O 90	++++	++++	+++±	−	−	−	+	±	+	++++	++++	+++
(2) K 158A	++++	+++	++±	±	±	−	+	±	+	++++	++++	+++
(3) K 107	++++	+++	+++±	±	±	±	−	±	+	+++±	++++	++±±
(4) K 127	*+	+	±		±	±	±	±	−	+	++++	+++
Type II strains												
(1) V 8	−	−	±	+++±	++++±	+++	±	±	0	++++±	++++	++++
(2) V 9	−	−	±	++++±	++++	+++	+	±	±	+	++++	+++
(3) C 69	−	−	±	++++	++++	+++	±	±	0	+++±	+++±	+++±
(4) B 92	−	−	±	+++±	++++±	++	±	−	+	+++±	++++	+++
(5) II 112	−	−	±	++++	+++±	±	+	±	0	++	+++±	+±±
(6) B 116	−	−	±	+++	++	+++	±	−	+	+++	+++	+++
(7) II 120	−	−	±	+++±	++++±	++	±	±	0	+++	+++	++±
(8) B 132	−	±	±	+++	++++	++±	−	−	−	+++	+++	+++±
Type III strains												
(1) M 216	−	−	±	±	−	−	+++	+++±	±	+++	++++	+++±
(2) K 151 A	−	±	±	±	±	−	++++	+++±	+	+	++++	+±±
(3) K 198	−	−	±	−	−	−	++++	++	±·+	+++	++++	++++
(4) B 63	−	−	±	±	±	−	++++	++	−	+++±	++++±	++++±
(5) B 115	−	−	−?	±	±	−	†+±±	±+	−	+++±	++++	++++
(6) B 135	−	−		±	±	−	†+	±	−	+++±	+++	++++±
Unclassified strains												
(1) B 125	−	±	±	±	−	−	±·	−	−	+++	+±±±	+++
(2) B 126	−	−	±	±	±	−	±·±	±	−	+++±	++++	++++±
(3) K 126	−	±	±	±	±	−	±	−	−	+++	+++	+++±

The following signs are used in all tables: ++++ to ± indicate degrees of reaction; − indicates a negative reaction; 0 indicates that the test was not made.

Controls with normal serum plus extract, and with serum plus saline, were all negative.

* Later experiments, described in the text, showed definitely that this strain belonged in Type I. † Concentrated extracts of these two strains gave as strong reactions with Type III antisera as any of the other members of Type III.

was only detectable after the reaction mixtures had stood overnight in the ice box. In nearly all instances the unpurified hydrochloric acid extracts gave entirely unequivocal results.

The three exceptional strains (K 127 in Type I, and B 115 and B 135 in Type III) seemed to contain minimal amounts of type-specific substance as shown by the slight precipitin reactions with antisera which proved later to be of the homologous type, and by the slight amount of type-specific antibody demonstrable in the serum of rabbits immunized with these organisms. Extracts of Strains B 115 and B 135 containing the type-specific substances concentrated by precipitation with alcohol were of sufficient potency to prove by the precipitin reaction that these strains belonged in Type III. The fact that Strain K 127 belonged in Type I was indicated by the use of similarly concentrated extracts and was further verified by preparing antisera with this organism which reacted specifically with a purified preparation of a known Type I polysaccharide and afforded mice protection against other strains belonging to Type I.

Similarly, the low content of type-specific substance may account for the difficulty in classifying Strains B 125, B 126, and K 126. This deficiency was suggested by the total lack of any demonstrable type-specific antibodies in the serum of rabbits immunized with these strains.

The classification shown in Table I was confirmed in numerous experiments by specific absorption of antibodies in the manner detailed under methods. An example is given in Table II.

One portion of Serum R 36-60, from a rabbit immunized with Strain O 90, Type I, was absorbed with heat-killed bacteria from a different Type I strain (K 158 A); and a second portion with bacteria from a Type II strain (C 69); a third portion was kept as a control. In this particular experiment, the serum was diluted with equal parts of physiological salt solution. Absorption was repeated a second time. The sera were then tested with several extracts of organisms of homologous and heterologous types, as shown in Table II.

The serum had a high titer of both group- and type-specific antibodies, as shown by the reactions of the unabsorbed serum with crude extracts of strains of all three types, by the reactions with a purified preparation of type-specific S substance of the homologous Type I strain, and by the reactions with the purified group-specific C substance. Two absorptions with a strain of homologous type (K 158 A, Type I) removed all antibodies from the serum. Similar absorptions with a strain of heterologous type (C 69, Type II) removed the group-specific antibody for C, as shown by the negative reactions with the crude extracts of Type II and Type III strains, and by the negative reaction with the purified group-specific substance, C. Absorption with this heterologous Type II strain did not, however, remove the antibody for the Type I specific polysaccharide, for the absorbed serum still reacted specifically with all preparations containing the Type I S substance.

In the course of these experiments, some difficulty and irregularity was encountered in the absorption of sera having a high concentration of antibodies for protein substances. Since, however, the antisera useful for type classification did not contain much antiprotein pre-cipitin, this difficulty is not significant in the work reported here.

The type differentiation above detailed was confirmed by passive protection tests in mice where virulent strains were available.

Only two of the 21 strains were sufficiently virulent for this purpose and these both belonged to Type I. They killed mice regularly in doses ranging from 10^{-6}

TABLE II

Absorption Experiment to Confirm Type Differentiation

Extract used in precipitin reaction	Type I serum, R 36-60		
	Unabsorbed control serum	Absorbed with Strain K 158 A Type I	Absorbed with Strain C 69 Type II
Type I Strains			
(1) O 90	+++	—	++
(2) K 158 A	+++±	—	++
(3) K 107	+++	—	+±
(4) K 127	++±	—	+±
Type II Strain			
(1) B 112	++±	—	—
Type III Strain			
(1) K 198	++±	—	—
Purified substances from Strain O 90 (Type I)			
(1) Type-specific (S substance)	++±	—	++
(2) Group-specific (C substance)	++++	—	—

cc. to 10^{-8} cc. of an 18 hour broth culture. All other strains were of such low virulence that even doses of 10^{-1} cc. did not kill mice, and in many instances 0.5 cc. was non-fatal.

The mouse protection tests were performed as follows: 0.5 cc. of serum was injected intraperitoneally into each mouse; the following day, 0.5 cc. of the appropriate dilution of a 16 hour broth culture, diluted serially in broth, was also injected intraperitoneally. All mice surviving the 10 day period of observation were considered effectively protected and were recorded as survivors. Estimations of the number of organisms in a given inoculum were made by plating in blood agar 0.5 cc. amounts of the three highest dilutions of the culture, and counting the colonies after 48 hours' incubation at 37°C.

The results of the protection tests against the virulent Type I strains (O 90 and K 158 A) are shown in Table III. Type I antiserum afforded protection to mice against 10 to 100 million fatal doses of

TABLE III

Cross-Protection Tests in Mice to Confirm Type Differentiation
The Two Virulent Type I Strains Tested with Sera of Homologous and Heterologous Types

Culture	Virulence controls		Antiserum against		
	No serum	Normal serum	Strain O 90 Type I	Strain V 9 Type II	Strain M 216 Type III
cc.					
*Strain O 90					
Type I					
10^{-8}	D 53 hrs.	—	—	—	—
10^{-7}	D 51 "	—	—	—	—
10^{-6}	D 48 "	D 41 hrs.	S	D 40 hrs.	D 41 hrs.
10^{-5}	D 48 "	D 41 "	S	D 65 "	D 41 "
10^{-4}	D 72 "	D 41 "	S	D 41 "	D 41 "
10^{-3}	D 21 "	D 42 "	S	D 41 "	D 23 "
10^{-2}	D 17 "	D 17 "	S	D 17 "	D 17 "
10^{-1}	D 17 "	D 17 "	S	D 17 "	D 17 "
† Strain K 158 A					
Type I					
10^{-8}	S	—	S	S	‡S
10^{-7}	D 42 hrs.	—	S	S	D 65 hrs.
10^{-6}	D 42 "	—	S	D 41 hrs.	S
10^{-5}	D 18 "	—	S	D 41 "	D 41 hrs.
10^{-4}	D 33 "	—	S	D 89 "	D 65 "
10^{-3}	D 18 "	—	S	D 41 "	D 160 "
10^{-2}	D 18 "	—	S	D 18 "	D 116 "
10^{-1}	D 18 "	—	S	D 17 "	D 18 "

S indicates survival for 10 days.
D indicates death within the number of hours indicated.
— indicates test omitted.
* Estimated by plate counts as 346 million colonies per cc.
† No colony counts made.
‡ For this test a Type III serum prepared against Strain B 63 was used.

cultures of the homologous type, while Type II and Type III antisera gave no protection whatever against these strains. In the control series, without serum or with normal serum, no protection was ob-

served. The Type I serum used in the experiment recorded in Table II was prepared by immunization of rabbits with Strain O 90. Similar tests with serum prepared with another strain of Type I (K 127) also resulted in protection of the mice.

It is interesting to observe that type-specific protection is effective in Group B when infecting organism and specific immune serum are injected simultaneously. With Group A organisms, in which the type-specific substance is a protein, it is necessary to inject the antiserum at least 8 hours before the infecting organism in order to demonstrate protection; but with Group B organisms, in which the type-specific substance is a polysaccharide, the simultaneous injection of the organism and its homologous antiserum results in specific protection of the mice, as is also true with certain other bacteria, such as the pneumococcus, in which a polysaccharide is the type-specific substance.

The Agglutination Reaction

The method of agglutination was used in an attempt to differentiate these Group B strains into types before the method of specific precipitation was adopted, but it was only partially successful on account of the particularly troublesome cross-agglutinations among these strains. While this difficulty might have been overcome by employing specifically absorbed sera in the agglutination reaction, it seemed more convincing, as well as easier, to use the direct precipitin test for the type classification rather than to rely entirely on the results obtained with absorbed sera. With the precipitin test, the types were sharply and unequivocally defined where certain antisera were used, although the agglutination test with these same sera yielded many confusing cross-reactions.

The agglutinations were performed by diluting the serum in infusion broth and mixing 0.5 cc. of serum dilution with 0.5 cc. of 18 hour broth culture. If the culture showed any tendency to agglutinate spontaneously, it was washed and resuspended in fresh broth before use. The tests were read after 2 hours' incubation at 56°C.

Table IV shows the results of the agglutination reactions of one strain in each type giving the most marked type-specific reaction, and of one strain giving the most marked cross-reaction. The sera were those used for type differentiation with the precipitin reaction (recorded in Table I). With most Type I strains, O 90 for example, there was very little cross-agglutination in the sera of other types,

TABLE IV

Cross-Agglutination Reactions

Culture	Type I					Type II							Type III					
Antiserum against / Final serum dilutions	1-20	1-40	1-80	1-160	1-320	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-20	1-40	1-80	1-160	1-320	1-640
Type I strains																		
(1) O 90	++++	++++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
(2) K 127	+++±	+++±	++±	+±	−	+±	+±	+±	−	−	−	−	++	±	−	−	−	−
Type II strains																		
(1) B 132	±	−	−	−	−	++++	++++	++++	++	++	++	++	++	++	++	+	−	−
(2) B 92	−	−	−	−	−	+++	+++	+++	−	−	−	−	+++	+++	++	−	−	−
Type III strains																		
(1) K 151	−	−	−	−	−	+	±	−	−	−	−	−	++++	+++	+++	+++±	++±	+
(2) B 63	++	++	±	−	−	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	+++	++±

Controls with broth and with normal serum in the same series of dilutions were negative.

although with such strains as K 127, containing minimal amounts of type-specific substance, considerable cross-reaction was observed. Because the Type I serum employed gave only slight cross-reactions with strains of other types, the agglutination test was of value in classifying the Type I strains. The agglutinated organisms coalesced into a compact disc, like that seen in type-specific pneumococcus agglutinations in contrast to the less compactly agglutinated mass usual in streptococcus agglutinations. A further similarity with pneumococcus agglutinations was the low agglutinin titer attained, usually about 1–80 and not often exceeding 1–320. Between Types II and III strains and sera there was a large amount of cross-reaction, so that it was impossible to classify most strains of these two types by the simple agglutination test. Thus, Strain B 132, the most specifically reacting strain of Type II, still showed considerable cross-agglutination in Type III serum. Among Type III organisms, Strain K 151 A gave specific reactions, but most other members of this type gave cross-reactions like that shown by Strain B 63. With these two types, especially Type III, the agglutinated organisms coalesced less firmly into discs than the organisms of Type I, and the agglutinin titers reached the high level commonly found among antisera for streptococci of other groups.

The sera used for these agglutinations were selected for their low content of non-type-specific antibodies, and did not give much cross-reaction when used in the precipitin test (Table I). In the agglutination reaction, however, which is a more sensitive test for antibody, even this low concentration of group antibodies was sufficient to give marked cross-reactions. Since other sera, containing a higher concentration of group antibodies gave much more cross-agglutination, this reaction seemed less well adapted than the precipitin test for the study of the classification and antigenic composition of these strains of hemolytic streptococci.

The Chemical Nature of the Substances Determining Group and Type Specificity

It may be recalled that type specificity among Group A strains depends upon bacterial proteins, the so called M substances (3). The occurrence, therefore, of type-specific carbohydrates, previously unknown among hemolytic streptococci, was of particular interest in the typing of Group B strains.

The formation of typical disc-like precipitates, so generally characteristic of the union between bacterial polysaccharides and their corresponding precipitins, was the first suggestion of the possibility that the reactive substances in this group

were carbohydrates rather than proteins. Disc formation was particularly marked with Type I strains, although physical conditions, such as the relative concentrations of the reactive substance and its antibody, and the amount of shaking which the mixture received during incubation, played an important rôle in determining the character of the disc. There was a slightly greater tendency toward the formation of coarse, loose discs with Type II strains, and only under optimal conditions did Type III strains form characteristic discs. Further investigation indicated, however, that the poorer disc formation probably resulted partly from a lower concentration of type-specific antibody, and partly from an admixture of other reactive substances, protein in nature, which combined with group antibody present in the serum. This was evident in tests made with serum from which all except the type antibody had been absorbed, after which the type-specific precipitates appeared as more characteristic discs.

Digestion experiments with trypsin also suggested the polysaccharide nature of these type-specific substances of members of Group B. Discs typical of polysaccharides still occurred after digesting the extracts with trypsin; and the type-specific reactivity of these extracts was not diminished by even 6 days of tryptic digestion.

This additional indication of the non-protein nature of the reactive substances led to the preparation and purification of the so called S substance for the purpose of chemical analysis. For comparison, the group-specific C substance, also present in the crude bacterial extracts, was separated and analyzed at the same time.

Since the culture fluids contained only small amounts of either substance, it was necessary to extract the bacteria after centrifugation from the 0.1 per cent dextrose infusion broth, in which they were grown. The bacterial sediment was extracted by heating with N/20 hydrochloric acid, as already described (1). The extract from the Type I culture, Strain O 90, was then purified by repeated precipitations with three volumes of 95 per cent ethyl alcohol. After dialyzing and drying, this preparation (O 90, S 6, in Table V) was analyzed chemically and found to be essentially of polysaccharide composition.[1] It gave negative protein tests with the biuret reaction and trichloracetic acid, but it gave a very strongly positive Molisch reaction. The total nitrogen, probably largely impurity, was 2.14 per cent; and reducing sugars, calculated as glucose, after acid hydrolysis were 76.8 per cent; the acid equivalent was 5230; the optical rotation was − 4.8°. The substance reacted in homologous immune serum in a dilution of at least 1–3,000,000.

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[1] I am greatly indebted to Dr. W. F. Goebel for much advice in the preparation of these substances, and to Dr. Goebel and Dr. F. H. Babers for all of the analyses, which they were kind enough to perform.

When the foregoing analysis was made, the existence of the carbohydrate, C, specific for all Group B hemolytic streptococci, was unknown. Following the discovery of this substance, the above preparation of Type I specific polysaccharide was retested serologically and found to be a mixture of the type-specific S substance and the group-specific C substance, as shown by the precipitin tests recorded in the last four columns of Table V.

These two substances were therefore separated from each other by fractional precipitation with alcohol of a concentrated solution of preparation O 90, S 6. The S substance was precipitated with two volumes of 95 per cent ethyl alcohol, while the C substance required a higher concentration of alcohol for complete precipitation. These two fractions were then dialyzed and dried as before. The analyses, given in Table V (O 90, S 6a, and O 90, C 6), show that both are in all probability polysaccharides. The fraction containing the S substance still gave a type-specific precipitin test in a dilution of at least 1–3,000,000, but no longer reacted with serum containing the group antibody. The fraction containing the C substance, on the contrary, gave no reaction with purely type-specific antisera, but gave a specific precipitate in a dilution of at least 1–4,000,000 with antisera rich in group-specific antibody.

The material from the Type II strain, V 9, was similarly extracted.

One lot, prepared by repeated precipitation with alcohol, yielded a small amount of very impure S substance; although the C substance, collected from the same lot, was more successfully purified. Its characteristics are recorded in Table V (V 9, C 1). A second lot of S substance was collected from Strain V 9 and purified by precipitating the impurities with trichloracetic acid, as suggested by Goebel (5). The S and C substances, freed of protein by this method, were then separated by fractional precipitation with ethyl alcohol. The analysis of the type-specific S substance from this preparation is given in Table V (V 9, S 3).

The data presented in Table V indicate fairly clearly that the group-specific C substance and the type-specific S substances of Types I and II are carbohydrate in nature. Due to the great difficulty in obtaining more than very small amounts of these substances, fuller chemical data are not available. For the same reason, no analysis was made of the S substance characteristic of Type III strains.

All of the S and C preparations analyzed (Table V) gave negative qualitative protein tests; most of them showed a total nitrogen content of approximately 2 per cent, part of which may be due to impurities. All gave very strong Molisch

TABLE V

Properties of Type-Specific S Substances and of Group-Specific C Substance

Strain	Preparation		Chemical analysis		Serological reactions			
	Lot No.	Type or group substance	Total nitrogen	Reducing sugars after hydrolysis (calculated as glucose)	Highest dilution of substance reacting in the precipitin test with			
					*Anti-S serum			*Anti-C serum
					Type I	Type II	Type III	Group B
			per cent	per cent				
O 90	†S 6	Mixture: S of Type I and C of Group B	2.14	76.8	1–3,000,000	–	–	1–100,000
O 90	S 6a	S of Type I	1.93	65.6	1–3,000,000	–	–	–
O 90	C 6	C of Group B	‡Not done	61.0	–	–	–	1–4,000,000
V 9	S 3	S of Type II	1.43	61.2	–	1–4,000,000	–	–
V 9	C 1	C of Group B	2.34	39.7	–	–	–	1–4,000,000

– indicates negative reaction.

* These were all antibacterial sera. Certain antisera rich in S antibodies and poor in C antibodies, were used for anti-S sera. Other antisera, rich in C antibodies, were used in titrating the C substances.

† This preparation proved to be a mixture consisting chiefly of type-specific S substance but containing an appreciable quantity of group-specific C substance. These substances were separated and analyzed as O 90, S 6a, and O 90, C 6.

‡ Insufficient material for this analysis. A 1–265 dilution was biuret-negative and was not precipitated by picric, trichloracetic, or sulfosalicylic acids. It gave a very strong Molisch reaction.

reactions, even in considerable dilution; and after acid hydrolysis the reducing sugars, calculated as glucose, varied from 40 to 77 per cent in the different preparations. The differences in the group-specific C substances in preparations from the strains representing Types I and II were presumably due to the presence of impurities, for, serologically, these two preparations were identical in their reactions. The S preparations, on the contrary, were serologically distinct in each type and reacted only with antisera of the homologous type.

To summarize, the chemical analyses, together with the non-digestibility with trypsin, and the formation of disc-like precipitates, seem sufficient evidence to characterize as polysaccharides the group-specific C substance and the type-specific S substances (Types I and II) isolated from members of Group B hemolytic streptococci.

<div align="center">DISCUSSION</div>

The data presented show that the hemolytic streptococci, differentiated into Group B by the serological methods described in a preceding paper (1) can be further subdivided into specific types. Although the agglutination reaction was found unsuitable for differentiation of types on account of the large amount of cross-agglutination, the precipitin reaction proved suitable for this classification under proper precautions. The principle involved was the production of immune sera in which the type-specific antibody was predominant. This was achieved, to a certain extent, by the particular method of immunization and also by the method of selective absorption of the antisera. Type-specific antisera thus prepared were used with bacterial extracts in the precipitin reaction to differentiate into specific types the 21 strains of Group B hemolytic streptococci which were available for this study. With three exceptions, these strains fell into three specific types. Passive protection tests in mice furnished confirmatory evidence of this specific type classification.

The purified type-specific substances obtained from bacterial extracts of representatives of Types I and II were analyzed chemically, and even though the small amount of available material precluded full chemical analyses, the data indicate that the type-specific substances belong to the class of carbohydrates. The evidence for their non-protein nature is further supported by the complete failure of trypsin to destroy them. Although a chemical analysis of the Type III

specific substance was not made on account of the difficulty of obtaining material, its resistance to prolonged tryptic digestion was considered sufficiently conclusive evidence of its non-protein nature.

For purposes of comparison with these type-specific polysaccharides (designated as S substances), the chemical analysis is also given of the substance (designated as C) which determines the group specificity of all members of Group B irrespective of type. As previously shown (1), substances identical in their serological reactions are obtainable from all members of Group B; and the data, while insufficient to establish the chemical identity of the C substances extracted from different strains, are, nevertheless, sufficient to show that the C substance in the different preparations is in every case of carbohydrate nature. Each member of Group B hemolytic streptococci contains, therefore, one polysaccharide, C, which determines its group specificity, and another polysaccharide, S, which determines its type specificity.

The recent work of Stableforth (6) also demonstrated specific types among streptococci of bovine origin. The strains studied by him were classified into groups on the basis of cultural and biochemical tests. The group containing all of the hemolytic strains in his collection also included a large number of non-hemolytic strains. He suggests, on the basis of certain observations made in the course of his work, the possibility of a change in hemolytic properties, but reserves the further consideration of such a possibility until more evidence has accumulated.[2] With the exception of the non-hemolytic strains, the biochemical and cultural properties of the streptococci designated by Stableforth as "Group I" correspond to those here included in Group B. Using the precipitin reaction, as well as Griffith's slide agglutination technique (7), with both absorbed and unabsorbed sera, Stableforth was able to classify the 91 strains of "Group I" into three distinct serological types. It is not known whether these types correspond to those found among the Group B strains studied here.

Stableforth's comprehensive study was not concerned with the chemical nature of the determinative type-specific substances, and he supposed, on account of the character of the precipitates formed at 50–55°C., that the type-specific substances were protein in nature, and similar to the type-specific substances of hemolytic streptococci of human origin. In view of the present study, it seems more probable, however, that the non-disc-like character of the type-specific precipitates observed by Stableforth was due, rather to physical factors, such as the relative concentrations of the reactive substance and antibody, or possibly the higher tem-

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[2] A report of a change in the hemolytic properties of one Group B strain included in the present studies will be the subject of a subsequent paper.

perature used in incubation, than to the presence of type-specific substances of protein nature.

A summary of the differentiation of hemolytic streptococci, thus far achieved, is given in Table VI, which also contains the results of the study of the chemical nature of the determinative substances in so far

TABLE VI

Summary of the Serological Differentiation of Hemolytic Streptococci into Groups and Types

Hemolytic strepto-cocci differentiated into groups by precipitation with group-specific substance, C, a polysaccharide	Chief source	No. of strains studied	Type specificity within the group determined by
Group A	Man	23*	Precipitation with type-specific protein, M, agglutination, and mouse protection. Many types
Group B	Cattle	21	Precipitation with type-specific poly-saccharide, S; and in some instances agglutination and mouse protection. 3 types include all but 3 of these strains
Group C	Various animals: cattle, guinea pigs, rabbits, horses, swine, chickens, foxes	49	Not well studied
Group D	Cheese	8	" " "
Group E	Certified milk	3	
Unclassified	Man, cattle	2	

* Many more strains of human origin have been tested and found positive for the presence of the C substance characteristic of Group A, but only 23 were used in the particular study (1) in which the group classification was made. Other Group A strains have also been classified as to type by the precipitin method (3, 8, 9).

as they are now known. It is evident that there exist groups (1) having more or less common animal origins, and that each group elaborates a carbohydrate substance (C) upon which the primary classification depends. The chemical nature of the substances responsible in any particular group for the further division into types is unpredictable and can only be determined experimentally. Previ-

ously (3) it was established that the serological differentiation into types among the members of Group A is determined by a protein (M). The present work, on the other hand, shows that the types existing among members of Group B are differentiated by carbohydrates specific for each type.

SUMMARY

1. Hemolytic streptococci of Group B (derived chiefly from cattle) have been further subdivided by use of the precipitin reaction into specific types.

2. With three exceptions, the 21 strains of Group B were differenated into three specific types.

3. Chemical analyses of the type-specific substances of Group B strains of Types I and II show that they are polysaccharides (S substances). This is in contrast to the fact that proteins (M substances) were previously shown to determine type specificity among strains of human origin (Group A).

4. The group-specific substance, C, serologically identical in all members of Group B, was also identified as of polysaccharide nature.

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LOSS OF THE PROPERTIES OF HEMOLYSIN AND PIGMENT FORMATION WITHOUT CHANGE IN IMMUNOLOGICAL SPECIFICITY IN A STRAIN OF STREPTOCOCCUS HAEMOLYTICUS

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The evidence presented in this paper shows that a strain of hemolytic streptococcus lost its functions of producing hemolysin and pigment but retained its serological specificity.

Valentine and Krumwiede (1) reported alterations in a culture of hemolytic streptococcus of human origin which resulted in a suppression of the power to elaborate hemolysin. Among numerous strains of hemolytic streptococci investigated by them one gave rise, after repeated subcultures, to about 10 per cent of non-hemolytic colonies which were surrounded by a green zone, while the remaining colonies were normally hemolytic. Subcultures from individual colonies of both forms remained constant in their effect on blood, but the organisms were, nevertheless, immunologically identical in so far as could be determined by immune sera prepared against pure cultures of each form. The immunological identity of the two strains was established by the reactions of agglutination and agglutinin absorption. The hemolytic form was slightly more virulent than the non-hemolytic, although in both instances the virulence was so low as to be of doubtful significance. The fact that mouse passage enhanced the virulence of the non-hemolytic form without restoring its hemolytic power indicated that the two functions were probably unrelated.

Todd (2) observed that the property of producing hemolysin disappeared in two strains of *Streptococcus haemolyticus* as the virulence of the organisms was increased by mouse passage. The formation of hemolysin by these strains was greatly influenced by growth under reduced oxygen tension. The virulent form, which was non-hemolytic in the presence of oxygen, was, when grown under anaerobic conditions, almost as hemolytic as the original avirulent organism. After many transfers on artificial media, the streptococci regained their hemolytic property and lost progressively their virulence for mice. This reversion, together with the fact that the cultures were always hemolytic in the absence of oxygen, was considered sufficient identification of the organisms as modified forms of *Streptococcus haemolyticus*. In a subsequent study of one of these strains, the immunological

identity of the two forms was established by type-specific (M) precipitin reactions and mouse protection tests (3).

A somewhat similar change to that observed by Todd as occurring during mouse passage was reported recently by Fry (4) as probably occurring in the human subject. In six cases of puerperal sepsis both hemolytic and green-forming streptococci were isolated in aerobic cultures from different foci of infection in the same patient. In four other cases only green-producing streptococci were obtained, while in one additional case a mixture of green-producing and hemolytic forms was found in several successive blood cultures from this patient. In addition, at least two cultures which were slightly hemolytic and one which was strongly hemolytic when first isolated, lost their hemolytic capacity and became green-forming after several subcultures. All of these strains produced hemolysin when grown anaerobically or when grown in 10 per cent horse serum broth. Serological studies, made to test the identity of the strains obtained from a single patient, were completed in two cases. In both instances, reciprocal agglutinin absorption experiments were strongly suggestive of the serological identity of the hemolytic and green-producing strains isolated from the same individual.

The loss of certain functions, recorded in this paper, occurred in *Streptococcus haemolyticus* O 90 during a serological study of this organism. As previously noted (5, 6), this strain of streptococcus was classified serologically as a member of one of the specific types of Group B, the group composed chiefly of strains of bovine origin. It may be recalled that a C substance, specific for Group B, is elaborated by all members of this group and serves in the precipitin reaction to identify strains included in the group. More complete identification is afforded by classification into types by means of type-specific polysaccharides, the so called S substances. The strain under discussion, Streptococcus O 90, possessed in addition the distinguishing characteristic of elaborating a yellow-brown pigment. For several years it has been observed that certain strains, particularly those other than human in origin, form pigments varying in color from yellow-brown to lemon. The pigment is contained within the bacterial cells and is manifest in microorganisms grown in fluid medium and also in plain agar plates without dextrose or with dextrose in concentrations lower than 1 per cent. It is apparent only under conditions of partial or of complete anaerobiosis, as in deep broth cultures, in the deep colonies in poured plates, or in cultures grown in anaerobic jars. Under aerobic conditions the surface colonies on plain blood agar plates are colorless.

This relationship between color and oxidized state of the pigment has not, however, held true with every chromogenic strain. Among 29 strains of hemolytic streptococci classified in Groups B and D, nine were observed which produced pigment varying in amount and color. Pigment-forming strains occurred in all three serological types of Group B.[1] Although two of the chromogenic strains were of human origin, the remaining seven were derived from sources other than human. One of the strains of human origin was obtained in pure culture from the throat of a child, but in spite of this gave rise to no symptoms of disease. The other strain with a history of human derivation had been under laboratory cultivation for many years. All of its cultural, biochemical, and serological reactions were the same as those of the other Group B strains of non-human origin.

A considerable quantity of pigment was obtained from Strain O 90. Following acid extraction of the bacteria, a layer of precipitated pigment was observed on the upper surface of the bacterial sediment. On neutralization of the solution in which the previously extracted bacteria were suspended, the pigment dissolved and could be separated by throwing down the bacterial sediment in a centrifuge. The water-soluble pigment was deep red and markedly fluorescent. It was precipitated on the addition of hydrochloric acid, and was redissolved on neutralization; it was not soluble in 95 per cent ethyl alcohol, in ether, or in acetone in this extracted form, nor could it be extracted from intact bacteria which were suspended in these solvents. The dissolved pigment did not hemolyze rabbits' red blood cells.

The only record found in which the occurrence of chromogenic hemolytic streptococci was noted was that of Durand and Giraud (7). The eleven strains, out of 125 examined, in which they observed a yellow-brown pigment were all of human origin except one isolated from a rat. Some of these streptococci were the causative agents of disease and others were considered to be saprophytes. The chromogenic strains in the present study, on the other hand, were chiefly non-human in origin, as noted above, and were all in serological groups not usually associated with human disease.

Durand and Giraud found that the pigment was formed in the absence of oxygen, and that impoverishment of the medium with respect to starchy constituents

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[1] The characteristics of these strains have been recorded in preceding papers (5, 6). Their distribution with respect to serological types was as follows: eight belonged to Group B: Strains O 90 and K 158 A were in Type I; Strains V 9, B 112, and B 132 were in Type II; Strains B 115 and K 198 were in Type III; and Strain B 126 was unclassified. Strain C 7 belonged to Group D.

resulted in diminution or disappearance of chromogenic power. Potato starch, glycogen, inulin, and dextrose favored the elaboration of pigment while certain other carbohydrates inhibited it. They found it difficult or impossible to restore the pigment-forming function after it had been suppressed. They state that they were able to demonstrate some serological relationship among the chromogenic strains by the reactions of agglutination and agglutinin absorption, but the details of these serological relationships are not reported. The pigment described by Durand and Giraud appears similar in its reactions to the pigment of Strain O 90 observed in the present studies.

The alterations in Strain O 90 occurred during the first or second passage of the culture through mice, but whether this procedure bore any causal relationship to the changes observed is undetermined. In subcultures in poured blood agar plates, made after the second mouse passage, a large majority of the colonies showed neither surrounding zones of hemolysis nor greenish discoloration of the blood. When preliminary serological tests demonstrated the improbability of the non-hemolytic variant being a contaminant, more extensive investigations were undertaken to establish the relationship of the two forms. It was observed, at once, that the non-hemolytic form did not elaborate pigment in either solid or liquid media when grown aerobically or anaerobically. There was, however no indication that hemolysin and pigment are the same substance, for in addition to numerous other differences, solutions of the pigment did not hemolyze red blood cells.

With the exception of hemolysin and pigment-producing properties, all cultural and biochemical characteristics of the variant were the same as those previously observed in the original organism (5, 6).

Thus, both forms attained a final hydrogen ion concentration in 1 per cent dextrose broth of pH 4.4, both hydrolyzed sodium hippurate and grew on bile agar plates, both fermented trehalose but not sorbitol, and both failed to reduce methylene blue in milk and were unaffected by streptococcus bacteriophage. Each form of this strain has, as far as observed, remained true to form for a period of almost 3 years under a variety of cultural conditions and during serial passages through mice.

Before the immunological tests were performed, the purity of the strains with respect to hemolytic properties was insured by cultivation in series with the selection of single colonies from blood agar plates. The two forms were then employed for preparing antisera.

Formalinized cultures, injected intravenously, were used as the immunizing agents. Rabbits R 32-61 and R 32-62 received the hemolytic form, and R 32-63 and R 32-64 the non-hemolytic form, in three weekly courses. The resulting serum was employed in agglutination, precipitin, and mouse protection tests.

Before use in the serological tests the variant and the original form of this strain were again tested for hemolytic and pigment-producing capacity, in both aerobic and anaerobic cultures. Tests for hemolysin in fluid cultures were also made in the usual manner by mixing broth cultures in varying dilutions with rabbits'

TABLE I

Cross-Agglutination Tests

Antisera and Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain O 90

Antiserum		Cultures used in agglutinations	Final serum dilution						
Against	Serum No.	Form	1-20	1-40	1-80	1-160	1-320	1-640	1-1280
Hemolytic form........	R 32-61	Hemolytic	++++	++++	++++	++++	+	-	-
" "	R 32-61	Non-hemolytic	++++	++++	++++	++++	+	-	-
" "	R 32-62	Hemolytic	++++	++++	++++	±	-	-	-
" "	R 32-62	Non-hemolytic	++++	++++	++++	±	-	-	-
Non-hemolytic form....	R 32-63	Hemolytic	++++	++++	++++	+	-	-	-
" "	R 32-63	Non-hemolytic	++++	++++	++++	+	-	-	-
" "	R 32-64	Hemolytic	++	+++	+++	+++	±	-	-
" "	R 32-64	Non-hemolytic	++	+++±	+++±	+++±	±	-	-

In Tables I, II, and III ±, +, ++, +++, ++++ indicate degrees of reaction; — indicates a negative reaction.

In the agglutination reactions in Tables I and III, broth controls and controls with normal serum in the same series of dilutions as the immune serum were completely negative.

washed red blood cells. The original culture maintained its hemolytic and chromogenic functions; the variant lacked them.

Agglutination tests were performed as follows: The bacterial cells, after centrifugation from 18 hour broth cultures, were resuspended in broth and added in volumes of 0.5 cc. to equal volumes of serum diluted serially in broth. Readings were made after incubation for 2 hours at 56°C.

The data presented in Table I demonstrate that an immune serum prepared with either form of this strain agglutinated both the original and the variant to the same titer. The non-hemolytic form agglu-

tinated somewhat more rapidly than did the hemolytic, but the final titers always corresponded and the agglutinated organisms in both instances took the form of compact discs. As a general rule, the agglutination reaction is not suitable for immunological identification of Group B hemolytic streptococci on account of the marked tendency towards non-specific cross-agglutination. However, this difficulty was not encountered in these experiments because organisms of Type I, to which these strains belong, agglutinate specifically. Consequently, the fact that reciprocal specific agglutination of both strains occurred was valid evidence in support of the view that they were immunologically identical.

Additional proof of this identity was furnished by precipitin reactions. It has been established that from hemolytic streptococci belonging to Group B two characteristic polysaccharides are extractable: (1) a group-specific C substance, and (2) a type-specific S substance. Since Strain O 90 belongs to Group B, Type I, it was possible to establish the serological identity of the non-hemolytic variant and the original hemolytic strain by testing for the presence of these two carbohydrates by means of the precipitin reaction.

Hydrochloric acid extracts (5) were made from the bacterial sediment of both varieties of organisms, using portions of the same cultures employed in the agglutinations. These extracts were tested for the group-specific C substance with an antiserum known to contain anti-C precipitin, but no Type I anti-S precipitin. The same extracts were tested for the Type I S substance by precipitin tests with the type-specific antisera used in the agglutination reactions.

The precipitin tests were performed as follows: To a series of tubes containing 0.4 cc., 0.1 cc., and 0.025 cc. of extract in a final volume of 0.4 cc., a constant volume of 0.2 cc. of serum was added. Final readings were made after 2 hours incubation at 37°C., and overnight in the ice box.

The results are summarized in Table II. It was evident from the results of the direct precipitin tests that the group-specific polysaccharide, C, and the type-specific polysaccharide, S, were both present in the variant of Strain O 90 and in about the same amounts as in the original hemolytic form.

The data presented in the third column show that extracts of the original and the changed form of Strain O 90 reacted equally well with a serum of known anti-C content. The precipitates were disc-like but not heavy because this antiserum

was of only moderate titer. In order to eliminate the type-specific S reaction and to bring out the group-specific C reaction, the antiserum employed to detect the C substance was prepared with a strain of a serological type heterologous to Strain O 90. On the other hand the relationships due to the type-specific S substance in these two strains are shown when extracts are tested with serum of the homologous type, as indicated in the last four columns of Table II. Sera from Rabbits R 32-61 and R 32-62, immunized with the hemolytic form of Strain O 90, and sera from Rabbits R 32-63 and R 32-64, immunized with the non-hemolytic form of

TABLE II

Cross-Precipitin Tests

Antisera and Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain O 90

Extracts		Anti-C serum (containing no Type I anti-S precipitins) against a Type III strain	Type I anti-S serum against			
			Hemolytic O 90		Non-hemolytic O 90	
Culture	Amount		Serum R 32-61	Serum R 32-62	Serum R 32-63	Serum R 32-64
	cc.					
Hemolytic O 90 (Type I)	0.4	++	++++	++++	++++±	+++
	0.1	++	++++	++++	+++	++++±
	0.025	±	+++	++±	+±	++
Non-hemolytic O 90 (Type I)	0.4	+	++++	++++	+++	+++
	0.1	++	++++	++++	+++	++++±
	0.025	++±	++	++	+±	+±

In the precipitin reactions in Tables II and III, the precipitates indicated by +++ and ++++ signs were in the form of unbroken discs. Controls of extract with normal serum and with saline alone were uniformly negative.

Strain O 90, gave equally good precipitates of the disc type with extracts of both the hemolytic and the non-hemolytic forms of the microorganism. Although these sera were not specifically absorbed, most of the reaction was undoubtedly due to the presence of the Type I specific antibody, for preliminary tests had shown them to be almost entirely devoid of anti-C precipitin.

Agglutinin and precipitin absorption experiments further substantiated the evidence for the serological identity of the two forms of Strain O 90.

Samples of serum from a rabbit immunized with the hemolytic form of Strain O 90, were mixed with equal parts of heat-killed bacteria packed by centrifugation

in a graduated centrifuge tube. One sample was absorbed with organisms of the hemolytic form and one with organisms of the non-hemolytic form of Strain O 90. After 45 minutes' incubation in a water bath at 37°C., the mixtures were centrifuged and the supernatant serum thus absorbed was used for agglutination and precipitin tests in the manner described above.

TABLE III

Absorption of Agglutinins and Precipitins by the Hemolytic and the Non-Hemolytic Form of Strain O 90

(a) *Absorption of Agglutinins*

Antiserum R 36-60	Culture used in agglutinations	Results of agglutinations Final serum dilutions			
	Form	1–10	1–20	1–40	1–80
Unabsorbed............................	Hemolytic	++++	++++	–	–
"	Non-hemolytic	++++	++++	–	–
Absorbed with hemolytic O 90.........	Hemolytic	–	–	–	–
" " " O 90.........	Non-hemolytic	–	–	–	–
" " non-hemolytic O 90....	Hemolytic	–	–	–	–
" " " O 90....	Non-hemolytic	–	–	–	–

(b) *Absorption of Precipitins*

Antiserum R 36-60	Polysaccharides from hemolytic O 90 used in the precipitin test	
	C (group-specific)	S (type-specific)
Unabsorbed..	+++	++±
Absorbed with hemolytic O 90.........................	–	–
" " non-hemolytic O 90	–	–

0.2 cc. of antiserum and 0.2 cc. of a 1–20,000 dilution of polysaccharide were used in these tests. Final readings were made after 2 hours at 37°C. and overnight in the ice box.

From Table III *a* it is evident that Serum R 36-60, which agglutinated to the same titer both varieties of Streptococcus O 90, lost all of its type-specific agglutinins after absorption with either variety of organism. A comparable removal of precipitins from the same absorbed serum was demonstrated (Table III *b*). Absorption with either form of the streptococcus removed both anti-C and anti-S precipitins.

Comparative virulence and cross-protection tests were also made with the variant and the original form of this strain as follows:

16 hour broth cultures were diluted serially with broth so that 0.5 cc. contained the amount of original culture required. Dilutions ranging from 10^{-1} cc. to 10^{-8} cc. of original culture were inoculated intraperitoneally into white mice of about 18 gm. weight. Other sets of mice were inoculated simultaneously with similar dilutions of the culture together with 0.5 cc. either of normal serum or of the sera

TABLE IV

Cross-Protection Tests in Mice

Antisera and Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain O 90

Culture	Virulence controls		Antiserum against			
	No serum	Normal serum	Hemolytic O 90		Non-hemolytic O 90	
			R 32-61	R 32-62	R 32-63	R 32-64
cc.						
*Hemolytic O 90						
10^{-8}	S	D 42 hrs.	S	S	S	S
10^{-7}	S	D 42 "	S	S	S	S
10^{-6}	D 19 hrs.	D 19 "	S	S	S	S
10^{-5}	D 67 "	D 42 "	S	S	S	S
10^{-4}	D 19 "	D 19 "	S	S	S	S
10^{-3}	D 19 "	D 19 "	S	S	S	S
10^{-2}	—	D 19 "	S	S	S	S
10^{-1}	—	D 19 "	D 19 hrs.	D 19 hrs.	D 19 hrs.	D 19 hrs.
†Non-hemolytic O 90						
10^{-8}	S	D 120 hrs.	S	S	S	S
10^{-7}	D 47 hrs.	S	S	S	S	S
10^{-6}	D 45 "	D 96 "	S	S	S	S
10^{-5}	D 69 "	D 96 "	S	S	S	S
10^{-4}	D 45 "	D 72 "	S	S	S	S
10^{-3}	D 27 "	D 216 "	S	S	S	S
10^{-2}	—	D 72 "	S	S	S	S
10^{-1}	—	D 72 "	S	D 10 days	S	S

S indicates survival for 12 days.

D indicates death within the number of hours recorded.

— indicates test omitted.

* Estimated by plate counts as 461 million colonies per cc.

† Estimated by plate counts as 401 million colonies per cc.

of rabbits immunized with the hemolytic or the non-hemolytic form of Strain O 90. The serum was injected simultaneously with the culture, for it was known (6) that mice infected with Group B streptococci are effectively protected by this method. The mice were observed for 12 days and animals surviving for this period were considered effectively protected and were recorded as survivors.

The number of streptococci in the three highest dilutions was estimated by mixing the respective dilutions with blood agar in plates and counting the colonies after 48 hours' incubation.

The data recorded in Column 2 of Table IV show the relative virulence of the two forms of Streptococcus O 90. While the minimal lethal doses of the infecting organism were practically the same, the hemolytic form uniformly killed mice from 12 to 24 hours earlier than the non-hemolytic form in the same dilution. In addition to the virulence test just described, other controls (Table IV, third column) were included to show that normal rabbit serum did not protect mice against infection with these cultures. Samples of the same immune sera which were used in the agglutination and precipitin tests, however, protected mice against infection with the original culture and its variant.

SUMMARY

1. A variant arising in a culture of hemolytic streptococcus was shown to have lost the properties of producing pigment and hemolyzing blood. Despite the loss of these two functions, it had in common with the strain from which it was derived certain other distinguishing biochemical characteristics, as follows: Both attained the same hydrogen ion concentration in dextrose broth; both hydrolyzed sodium hippurate, grew on bile agar, and fermented trehalose but not sorbitol; both failed to reduce methylene blue in milk cultures, and were insusceptible to the action of streptococcus bacteriophage. In addition, the virulence of the variant remained the same as that of the original culture.

2. The antigenic and serological specificity of the variant was identical with the group and type specificity of the original strain (Group B, Type I). These specificities were established by the use of immune sera prepared by immunization of rabbits with each form. The immunological reactions employed were reciprocal agglutination, precipitation, agglutinin and precipitin absorption, and passive mouse protection.

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STUDIES ON TYPHUS FEVER

XII. The Passive Immunization of Guinea Pigs, Infected with European Virus, with Serum of a Horse Treated with Killed Rickettsia of the Mexican Type[*]

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In the preceding paper of this series (No. XI) (1), we reported that we had succeeded in preventing the development of typhus fever in guinea pigs inoculated with virus of the Mexican type by subcutaneously administering serum of a horse immunized with homologous killed *Rickettsiae*. Protection was obtained even when the subcutaneous serum injections were made as late as 72 hours after intraperitoneal administration of the virus. The prophylactic possibilities of this serum were suggested by experiments which showed that complete or partial prevention of subsequent infection could be attained when virus was administered 1, 7 and 13 days after serum injections. Prophylactic effects had, however, completely disappeared on the 18th day under the conditions and dosage employed at that time. This phase of our work has, since then, been confirmed by Varela (2) who, using serum sent to him by us, and employing a somewhat larger dosage (*i.e.* 2 cc. subcutaneously), found that guinea pigs so treated were protected for 15 but not for 30 days. The implications of these experiments for practical prophylactic purposes are obvious if one considers that infected lice die within 10 to 12 days.[1]

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[1] The therapeutic possibilities of the serum in man cannot, of course, be appraised by the guinea pig experiment. However, we have treated, with encouraging results, two severe cases in Boston in which the serum was given only from a sense of obligation because of the severity of the condition; and a Mexican govern-

At the time when the experiments referred to above were carried out, the serum of the *Rickettsia*-immune horse agglutinated the Weigl louse vaccines in dilutions of 1–640, and gave Weil-Felix reactions in dilutions of 1–320. The agglutinative values of the serum for our own Mexican vaccines could not be determined, since both inoculum and subsequent *Rickettsia* suspensions contained small amounts of rat protein, a circumstance which would have rendered agglutinations valueless. It is of interest to note that long and intensive treatment was necessary before the horse serum attained such values. We attribute this to the relatively small amounts of specific antigen which our earlier crude methods of vaccine production yielded. With the present X-ray rat method, which, incidentally, has been successfully continued for over a year, we believe that horse immunization will be less difficult.[2]

In spite of the potent agglutinating power of our horse serum for the *Rickettsiae* of the European disease, we were not able, at the time of our last report, to obtain any corresponding passive protection against the European type of virus. Such failure was difficult to interpret because of the many experimental facts which, in earlier work, had indicated the close antigenic overlapping of the two types of virus. Moreover, the agglutinating values mentioned above constituted valuable direct confirmation of our views of the close relationship between the two responsible organisms.

Believing that the failures of passive protection against the European type might be attributable to purely quantitative difficulties, we decided to continue treatment of the horse throughout the summer, in the hope of increasing the potency of the serum. This was done, and bleedings taken in the early autumn showed that the Weil-Felix

ment Commission has used the serum, to date, in 17 cases. The number is entirely too small to permit of any kind of conclusion, though an analysis of the Commission's work, which will shortly be published, is encouraging as far as it goes.

[2] The X-ray method seems to have given trouble to a number of investigators who have tried to repeat it. This we believe is due to insufficient radiation in most cases. Moreover, a certain amount of clinical experience with rats is necessary for judging the best time for autopsy of the animals. Properly carried out, the method yields large amounts of *Rickettsiae* with considerable regularity.

reaction had then attained about double its previous potency; that is, there was complete agglutination in dilutions of 1–640. We were unable to obtain a further supply of Weigl vaccines at this time, and could not therefore, determine whether reactions with the louse *Rickettsiae* had likewise increased.

In carrying out experiments with this somewhat more potent serum, we took into consideration the following circumstances: When one wishes to inoculate considerable doses of European virus, this is best accomplished by injecting mixtures of defibrinated blood and brain suspension taken from animals with active typhus on the 10th to the 14th days after inoculation. In such materials, *Rickettsiae* cannot be found, and are probably scarce and entirely intracellular. And since the infected cells are homologous, one is probably setting up, within the peritoneum, a temporary tissue culture from which any considerable discharge of *Rickettsiae* takes place only 4 or 5 days after injection when some of the infected cells have died.[3] By that time, serum injected together with inoculum may have been, in part, eliminated. In carrying out our protection experiments, we therefore allowed from 2 to 3 days to elapse between the intraperitoneal virus injection and the subcutaneous administration of the horse serum.

EXPERIMENTAL

Experiment I.—In a preliminary experiment, 4 guinea pigs received large intraperitoneal doses of European virus. After 4 days, 2 of them were given, subcutaneously, 2 cc. of the immune horse serum, and a further 0.5 cc. was given on the 5th day. The results were encouraging, since the 2 controls developed typical and severe typhus, while the treated animals showed nothing more than slight, temporary rise in temperature. No normal horse serum controls were done, since this experiment was carried out for purposes of orientation.

In the following three experiments, untreated controls and controls with normal horse serum were employed.

Experiment II.—All the 13 guinea pigs were intraperitoneally inoculated with European typhus virus in the form of brain emulsion mixed with defibrinated blood, the materials being taken at the height of the disease. 72, 96 and 120 hours after infection, 8 of these animals were each given subcutaneous injections of 1 cc.

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[3] We believe that this fact explains, in part, the longer incubation time observed with the European type in guinea pigs.

of immune horse serum.   3 controls were given normal horse serum at the same intervals, and in the same manner.   2 were left untreated.   We have charted these experiments by a simple method which takes up little space, without sacrificing clearness.   The caption under the chart is fully explanatory.
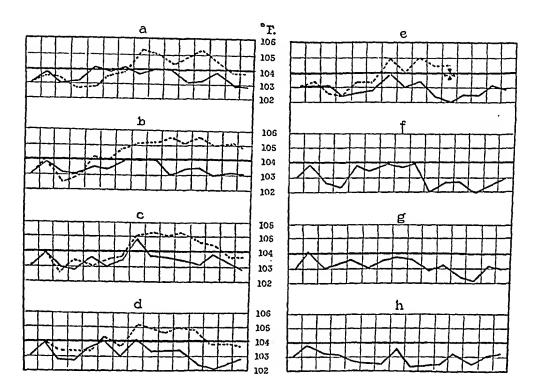
CHART 1. All of the 13 guinea pigs intraperitoneally inoculated with European typhus fever.   The 8 animals whose temperatures are charted in solid lines were given 1 cc. of the immune serum subcutaneously 72, 96 and 120 hours after infection.   Those charted in broken lines in Graphs a, b and c received normal serum in the manner and in the quantities in which the solid line animals received immune serum.   The guinea pigs charted in broken lines in Graphs d and e were untreated controls.   Horizontally, all the graphs represent daily temperatures for 15 days. The heavy line marking 104°F. is at the level above which we regard a guinea pig's temperature as indicating fever.

This experiment indicates that in the 8 treated guinea pigs, European typhus infection was prevented by subcutaneous immune serum injections which were not begun until 3 days after the virus had been administered.   The normal serum controls prove that this effect was not non-specific.

Experiment III.—The experiment shown in Chart 2 was done in exactly the same manner as the preceding one. The caption under the chart is sufficiently explanatory.

We were quite convinced that the results obtained in the two preceding experiments could not have been due to coincidence, since fortuitous failure to obtain typical reactions with this European strain has occurred on only rare occasions during the last 2 years of constant observation. However, in a fourth experiment of the same
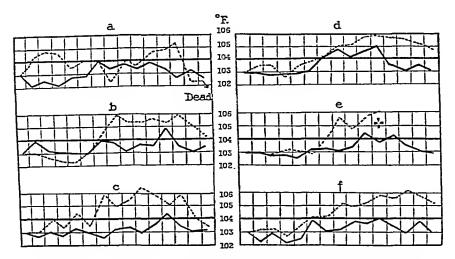
CHART 2. All 12 guinea pigs were intraperitoneally infected with European typhus virus on the same day. The solid lines in every case represent the temperature curves of infected guinea pigs which each received 1 cc. of antityphus horse serum 72, 96 and 120 hours after infection. The broken lines in Graphs *a*, *b*, *c* and *d* represent normal serum controls, and in Graphs *e* and *f* untreated controls. These graphs are constructed on the same plan as the two preceding ones. Horizontally the graphs represent daily temperatures for 15 days.

kind we had confusing results, which were readily explained by intercurrent disease. 6 animals were used in this experiment, all of them, owing to difficulties of animal stock, small and in poor condition. One of them died, but was not autopsied. 3 of the other 5 were killed when quite sick and on being autopsied showed pneumonias. Although there was ample reason, therefore, to exclude the atypical temperature reactions obtained in this experiment from any bearing on the effec-

tiveness of the serum, we decided to carry out one further experiment in order to make sure that we had not been the victims of coincidence in the preceding ones.
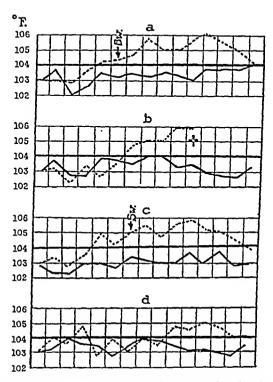
CHART 3. Again the solid lines represent the treated animals, the broken lines the controls.   In Graph *a*, the treated animal received 2 cc. of immune serum subcutaneously on the 2nd day and 1.5 cc. on the 4th day after infection.   The control received normal serum in the same amounts at the same intervals.   In Graph *b*, the solid line animal was treated as above, the other was untreated.   In Graph *c*, the treated animal received 2 cc. of serum subcutaneously on the 3rd day and 1.5 cc. on the 5th day.   The control received normal serum at the same intervals and in the same manner.   In Graph *d*, the treated animal received serum exactly as did the analogous one in Graph *c*.   The control was untreated.   Sw. indicates that the animals developed scrotal swelling—see comments in text. Horizontally, the graphs represent daily temperatures for 15 days.

Experiment IV.—This last experiment is presented in Chart 3.

These results differ from those of Experiments II and III only in the fact that the serum was given in somewhat larger quantities, but was started later; that is, 3 days after infection.   The results are entirely

clear and unambiguous, and together with those shown in Chart 2 seem to leave no room for doubt that the serum produced in a horse by treatment with Mexican *Rickettsiae* has exerted definite protective effect against the European typhus.

It is interesting to note that in Chart 3, Experiment IV, the 2 controls that received normal serum subcutaneously developed scrotal swelling. The increasing frequency with which the European strain is producing scrotal swelling is of considerable significance for the problem of the relationship between the two types of typhus fever. These two instances of swelling in animals receiving normal horse serum are noteworthy in view of Varela's (3) recent studies on the induction of scrotal swelling in animals infected with the Tunisian strain by the intraperitoneal injection of fresh sterile blood.

DISCUSSION

The experiments above recorded demonstrate that under suitable experimental conditions guinea pigs can be protected from infection with the European type of virus by the serum of a horse immunized with killed *Rickettsiae* of the Mexican type.

Apart from any practical implications which obviously encourage therapeutic test, these results seem to us of considerable bearing on the differences of opinion that exist concerning the closeness of the relationship between the two types of virus. It is our opinion that the differences are much less fundamental than they have been supposed to be, and that it is not impossible that they depend largely upon minor modifications (adaptations, possibly reversible) sustained in the course of the passage of the Mexican, so called New World virus through rats and rat fleas. A summary of the evidence in favor of such a view will be published at a later date.

The results of these experiments—showing beyond question that the serum of a horse treated entirely with Mexican *Rickettsiae* acquires a Weil-Felix reaction, agglutinates the European louse vaccines of Weigl and protects against the European virus—strongly support the view which closely relates these two infectious agents.

From the point of view of developing protective serum for prophylactic and therapeutic application to the European disease, these results are of importance because, so far, it has not been possible to obtain,

with the European virus, anything like the large accumulations of *Rickettsiae* which we have been able to produce with the Mexican strain by our X-ray method.

CONCLUSION

Guinea pigs infected with European typhus virus can be passively protected with the serum of a horse that has been treated with killed Mexican *Rickettsiae*.

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ON THE USE OF ADSORBENTS IN IMMUNIZATIONS WITH HAPTENS*

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Immunization with so called haptens, *i.e.* substances with definite serological activity but which with the usual laboratory methods exhibit rather weak or no immunizing capacity, was accomplished by Landsteiner and Simms (3), who obtained strong heterogenetic antisera following the injection of mixtures of Forssman's substance with human or pig serum. This method, which proved to be of general applicability, tended to emphasize the rôle of proteins in the production of immune sera to haptens, and under this assumption also showed that an immunization effect can be obtained without a firm chemical combination of hapten and protein.

Following this and the demonstration by Glenny (4), Ramon (5), and others that immunizations with toxins can be improved by adsorption to inert colloids, or the simultaneous injection of irritating substances, Gonzalez and Armangué (6) reported the interesting observation that extracts containing the heterogenetic substance can be made antigenic by adsorption to such materials as kaolin or charcoal. Doerr and Hallauer (7) had previously failed to immunize with mixtures of the heterogenetic extracts and charcoal. From this work of Gonzalez and Armangué it would appear that proteins can be effectually replaced by inert inorganic adsorbents in immunizations with haptens. The experiments of Gonzalez and Armangué have been confirmed by Landsteiner and Jacobs (1) with the Forssman antigen and recently by Plaut (8) with a hapten from brain.

In addition Zozaya (9) has reported considerable immunization effects with carbohydrates adsorbed to collodion particles and similar experiments with dextran, a carefully purified, nitrogen-free

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* The experiments given in this paper have been presented preliminarily by Landsteiner and Jacobs (1, 2).

polysaccharide from *Leuconostoc mesenterioides.*

On the other hand, with a carbohydrate from rhinoscleroma bacilli adsorbed to collodion particles by Zozaya's method, Prášek (10) failed to obtain evidence of antibody formation.

The following experiments were undertaken with the object of obtaining additional information on the mode of action of inorganic adsorbents in hapten immunizations.

Technique

Horse Kidney Extract.—Two extracts were used; one made with 95 per cent alcohol at room temperature, and the other by heating for 1 hour on the steam bath. For immunizing with kaolin 5 cc. of the crude heterogenetic extract (extract from 250 gm. of horse kidney in 85 cc. of saline) diluted 1:4 with saline was mixed with 0.2 gm. of kaolin, and allowed to stand at least an hour at room temperature, before each injection.

For immunizing with collodion particles 50 cc. of the crude heterogenetic extract diluted 1:4 with saline was added to sedimented collodion particles in a quantity of approximately five billion to each cubic centimeter of the extract. After thorough mixing, standing for 1 hour at room temperature, and centrifuging, the centrifugate was made up to 200 cc. with saline and 0.25 per cent phenol added.

Forssman Substance A.—This was identical with substance A described by Landsteiner and Levene (11), giving a strong orcinol-copper test. For immunizing with kaolin, a solution of 150 mg. of the substance in 150 cc. of phenolized (0.25 per cent) saline was added to 6.0 gm. of kaolin. The mixture was allowed to stand an hour at room temperature with vigorous shaking at short intervals. For immunizing with pig serum 150 mg. of the Forssman substance were dissolved in 127.5 cc. of saline, 15 cc. of pig serum and 7.5 cc. of 5.0 per cent phenol added, and the solution well mixed.

Forssman Substance B.—Substance B was a preparation obtained by precipitation with copper sulfate in alkaline solution (11). For immunizing with kaolin 35 mg. of the preparation were dissolved by the addition of 17.5 cc. of 5.0 per cent phenol and gentle warming. This solution was diluted to 35.0 cc. with distilled water, 7.0 gm. of kaolin added and made up to a volume of 175 cc. with saline, followed by vigorous shaking at 5 minute intervals for a period of an hour. In immunizations with pig serum, 30 mg. of the Forssman preparation were dissolved in 7.5 cc. of 5.0 per cent phenol and added to a solution containing 18.7 cc. of pig serum and 123.8 cc. of saline.

Specific Substance from V. cholerae.—The organisms were grown, extracted, and treated with N/10 NaOH as described previously (12). The resulting substance gave a distinctly positive biuret reaction at a dilution of 1:50. For injections, 90.0 mg. of the substance were dissolved in 9.0 cc. saline, 6 gm. of blood charcoal

added, phenolized (final concentration 0.25 per cent), and made up to a volume of 150.0 cc. with saline. The mixture was allowed to stand 1 hour at room temperature, with vigorous shaking at 5 minute intervals. A precipitation test on the supernatant of this preparation, after centrifuging, showed that the adsorption was almost complete.

Heteroalbumose.—This preparation was obtained by peptic digestion of coagulated sheep serum, as described previously (13). It was soluble in saline. For injections with charcoal 100 cc. of a 1.0 per cent sheep heteroalbumose solution in saline were added to 20.0 gm. of blood charcoal and the suspension thoroughly mixed and allowed to stand ½ hour at room temperature. 10 cc. of 5.0 per cent phenol were added and the volume made up to 200 cc. with saline. For injections with alum, to 100 cc. of 1.0 per cent sheep heteroalbumose solution in saline were added 10.0 cc. of 10.0 per cent alum potash followed by enough normal sodium carbonate to make the reaction slightly alkaline to litmus. The suspension was made up to 190 cc. with saline and 10.0 cc. of 5.0 per cent phenol added.

Adsorbents.—Acid-washed, biuret-free kaolin (Eimer and Amend) was used in these experiments, after washing with hot saline solution. Blood charcoal (Merck) was heated in a crucible and ground in a mortar before using. Collodion particles were prepared according to the method of Loeb (14).

Immunization.—For experiments on the production of hemolysins, rabbits were selected whose sera did not hemolyze sheep blood distinctly in a dilution of 1:25, under the conditions of the tests. Injections of 5.0 cc. each intravenously or intraperitoneally were administered at weekly intervals and the animals tested 7 days after the last injection. Injections of control solutions were always made with the same concentration and volume of solution, as was used with the adsorbents. Preparations with pig serum and collodion particles were injected intravenously; all other injections were intraperitoneal.

Precipitin Tests.—To 0.2 cc. of the antigen dilution in a small tube was added 2 drops of immune serum, and readings taken after 1 hour at room temperature, and overnight in the ice box. The strength of precipitation was recorded as follows: 0, f. tr. (faint trace), tr. (trace), ±, ∓, +, +±, ++, etc.

Agglutinin Tests.—To 0.5 cc. of the given dilutions of inactivated serum was added 0.5 cc. of a saline suspension of killed cholera vibrios grown overnight on agar slants. The tests were read after 2 hours at 37°C. and overnight in the ice box, and the same symbols used in recording the readings, as in the precipitation tests. Sediment was described as sl. (slight), w. (weak), or dis. (distinct).

Hemolysis Tests.—To 0.5 cc. of saline dilutions of each serum was added 0.5 cc. of fresh guinea pig complement, diluted 1:10, which did not cause hemolysis, under the conditions of the test. 1 drop (about 0.05 cc.) of 50 per cent washed sheep erythrocytes was added and the tubes incubated for 1 hour at 37°C. Degrees of hemolysis were distinguished according to the following scheme: 0, tr. (trace), w. (weak), dis. (distinct), str. (strong), v. str. (very strong), a.c. (almost complete), c. (complete).

EXPERIMENTAL

The experiments of Gonzalez and Armangué were repeated without difficulty,[1] using emulsions of horse kidney extracts, as shown in Table I. A marked difference in the action of the preparations adsorbed to kaolin as compared to the controls was noticeable even after the second injection.

TABLE I

Crude Horse Kidney Extract.   Tests Made after Five Injections

Material injected..............................	Horse kidney extract				
Rabbit No..........................	26-57	26-60	26-62	26-67	26-73
Complete hemolysis to................	<1:25	<1:25	<1:25	<1:25	<1:25
Material injected............................	Horse kidney extract adsorbed to kaolin				
Rabbit No.........................	26-59	26-64	26-66	26-71	26-73
Complete hemolysis to................	1:25	1:400	1:1600	1:25	1:200

TABLE II

Crude Horse Kidney Extract.   Tests Made after Five Injections

Material injected............................	Unheated horse kidney extract adsorbed to collodion particles				
Rabbit No..........................	18-94	18-95	18-96	18-97	18-98
Complete hemolysis to..............	<1:25	<1:25	<1:25	<1:25	1:50
Material injected............................	Heated horse kidney extract adsorbed to collodion particles				
Rabbit No..........................	18-99	19-00	19-01	19-02	19-03
Complete hemolysis to................	1:50	1:50	1:200	1:25	1:50

Although this was not the case in experiments with kaolin, with apparently less suitable adsorbents, such as collodion particles, crude heterogenetic extracts seemed to give better results when prepared by heating on the steam bath then when the extraction was performed at

[1] Weil and Berendes (15) who, like Misawa (16), reported negative experiments, note that their failure was associated with a heavy loss of animals due to the injection of kaolin. We, also, observed fatalities with injections of 0.5 gm., but had no difficulty with injections of 0.2 gm.

room temperature (Table II). In both cases, the fact that adsorption took place was shown by lysin inhibition using the adsorbed collodion particles and supernatants. With turpentine, injected intraperitoneally in a quantity of 0.1 cc. immediately following the injection of the Forssman extract, there was a similar but still less marked result.

Unheated extracts injected alone gave rise to practically no

TABLE III

Forssman Preparation A. Tests Made after Three Injections

Material injected............................	Forssman preparation A adsorbed to kaolin (orcin substance)				
Rabbit No...........................	30-86	31-51	31-52	31-53	31-54
Complete hemolysis to...............	<1:25	<1:25	<1:25	<1:25	<1:25
Material injected............................	Forssman preparation A mixed with pig serum				
Rabbit No...........................	30-84	30-85	31-48	31-49	31-50
Complete hemolysis to...............	1:800	1:50	1:100	1:100	1:400

TABLE IV

Forssman Preparation B. Tests Made after Five Injections

Material injected............................	Forssman preparation B adsorbed to kaolin				
Rabbit No...........................	19-04	19-05	19-06	19-07	19-08
Complete hemolysis up to............	<1:25	<1:25	<1:25	<1:25	<1:25
Material injected............................	Forssman preparation B mixed with pig serum				
Rabbit No...........................	29-10	29-11	29-12	29-13	29-14
Complete hemolysis up to............	1:800	1:200	1:800	1:800	1:320

hemolysin formation, while heated extracts alone yielded sera which were apt to give complete hemolysis at dilutions of 1:25 after four or five injections, but not in higher dilutions. An experiment in which heterogenetic antigen was injected intravenously and the usual amount of kaolin intraperitoneally, failed to show activation.

With the Forssman preparations designated as A and B which contained smaller quantities of extraneous material than the crude ex-

tract, no hemolysin formation was obtained, using kaolin.   As is evident from Tables III and IV, however, these same preparations, when mixed with pig serum, were active in producing hemolytic sera.

Adsorption to kaolin was tested by lysin inhibition with the adsorbed kaolin preparations and the supernatants.   Both Forssman substances

TABLE V

Crude Cholera Carbohydrate.   Tests Made after Five Injections

Injected with	Rabbit No.	Agglutination, serum diluted						Precipitation Dilution of antigen 1:5000	Read after
		1:50	1:100	1:200	1:400	1:800	1:1600		hrs.
Cholera preparation alone	26-46	0 / sl. sed.						0 / 0	2 / 24
	26-47	0 / dis. sed.						0 / 0	2 / 24
	26-48	0 / dis. sed.						0 / 0	2 / 24
Cholera preparation adsorbed to charcoal	26-49	+± / +±	+ / +	± / tr.	± / dis. sed.	tr. / w. sed.	f. tr. / sl. sed.	+± / +±	2 / 24
	26-50	+ / tr.	tr. / dis. sed.	0 / w. sed.	0 / 0			tr. / ±	2 / 24
	26-51	tr. / dis. sed.	0 / sl. sed.	0 / 0	0 / 0			f. tr. / f. tr.	2 / 24
	26-52	± / ±	tr. / dis. sed.	0 / w. sed.	0 / sl. sed.			tr. / tr.	2 / 24
	25-43	tr. / w. sed.	0 / sl. sed.	0 / 0	0 / 0			f. tr. / f. tr.	2 / 24

were largely adsorbed.[2]   Preparation A was administered in injection doses of 5 mg. each, and preparation B in amounts of 1 mg. per

[2] Adsorption of Forssman antigen to kaolin has been studied by Fischer (17), Weil and Berendes (15), Rudy (18), and others.

injection. With the latter substance a second experiment confirmed the first. With a third Forssman preparation (19), adsorbed to kaolin, attempts at immunization failed in a similar manner. These experiments furnish evidence that the injection of kaolin alone does not lead to the production of hemolytic sera, which is worth mentioning because Misawa (16) reported the production of hemolytic sera following injections of charcoal. He states, however, that his results

TABLE VI

Sheep Heteroalbumose. Precipitin Tests Made after Eight Injections

Substance injected	Rabbit No.	Heteroalbumose 1:2000	
		2 hrs.	24 hrs.
Heteroalbumose	29-30	0	0
	29-31	0	0
	29-32	0	0
	29-33	0	0
	29-34	0	0
Heteroalbumose adsorbed to alum	29-35	tr.	±
	29-36	0	tr.
	29-37	tr.	+
	29-38	tr.	±
	29-39	0	0
Heteroalbumose adsorbed to charcoal	29-40	f. tr.	±
	29-41	0	tr.
	29-42	±	+
	29-43	tr.	+
	29-44	+	+±

were uncertain, due to the possible presence of a *lepisepticus* infection in the rabbits.

With a cholera carbohydrate preparation which still gave a positive biuret reaction, adsorbed to charcoal, there was a marked increase in antigenicity over that shown by the carbohydrate preparation alone, as is evident in Table V. The controls in the agglutination test were not entirely negative, since they showed on standing overnight in the ice chest slight to distinct sedimentation after the third and subsequent injections.

With a carbohydrate preparation from the same organism which had been treated similarly with alkali but from which the protein was removed almost completely by heating with acetic acid so that the biuret reaction was but very faintly positive, one experiment with charcoal yielded no evidence of antibody formation whatever, and a second, one slight and one faint reaction. Five rabbits were used in each of these experiments.

Two preparations of dextran[3] adsorbed to collodion particles and injected by Zozaya's technique, failed to produce demonstrable antibodies. Two other entirely or practically protein-free carbohydrates, one from Pneumococcus Type I, and the other from a pseudoanthrax bacillus, adsorbed to charcoal and injected into rabbits, gave negative results. Thus attempts to demonstrate an increase in precipitin or agglutinin formation following injections of carbohydrates which had been freed, to a large extent, from protein, were not successful.

The heteroalbumose preparation used was known (20) to be a weak antigen with which sera of workable titer could be obtained only with difficulty. In the experiment shown in Table VI, comparable injections of heteroalbumose, alone and adsorbed either to charcoal or to alum, showed markedly increased antibody formation with the use of these adsorbents, which has been of practical advantage in the preparation of antisera to this material (21). In this experiment, the sera of the control rabbits, injected with heteroalbumose alone, happened to be entirely negative.

<div align="center">DISCUSSION</div>

In confirmation of the experiments of Gonzalez and Armangué we obtained very active heterogenetic immune sera with the aid of adsorbents. The enhancement of immunizing activity which takes place after adsorption to kaolin occurs whether the horse kidney extracts used are prepared by heating or by standing at room temperature. In the inconstant activations which followed adsorption to collodion particles or the simultaneous injection of turpentine, only the heated extracts showed a slight enhancement. These latter extracts were more highly colored than those obtained by standing at room tempera-

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[3] For supplying this substance we are indebted to the kindness of Dr. Harold Hibbert of Montreal.

ture, and caused slight hemolysin formation when injected alone, suggesting that they may contain more impurities than the unheated ones.

Experiments carried out with certain fractions of the original extracts gave results which seem to offer information on the activation by adsorbents. Forssman preparations which had undergone treatments designed to remove inactive material, although highly active in tests with immune sera, were no longer able to produce hemolysins on adsorption to kaolin. On the other hand, hemolysins appeared when these substances were injected along with pig serum. With this hapten preparation, therefore, we did not succeed in substituting kaolin for foreign serum, and the most obvious explanation would appear to be that some impurity is active in the enhancement of antigenicity by adsorbents. In regard to this it is of interest to note that Plaut (8), who was able to produce immune sera by the injection of brain hapten adsorbed to aluminum hydroxide, did not succeed in obtaining such an effect with a chemically pure substance, namely cholesterin, with which, however, antibodies can be produced, according to several authors, by admixture with antigenic protein.

The experiments with carbohydrates point in a similar direction. A preparation obtained from *V. cholerae* by extraction with 75 per cent alcohol and treatment with alkali, gave a strong biuret reaction and moderately strong agglutinin and precipitin formation in rabbits after adsorption to charcoal. Further treatment with protein precipitants resulted in a substance giving a negative or exceedingly faint biuret, and of very slight immunizing activity after adsorption. Carefully purified carbohydrates from *B. pseudoanthracis* and Pneumococcus Type I adsorbed to charcoal, and two different preparations of dextran[4] adsorbed to collodion particles and to charcoal, gave practically negative results. It would seem that with the methods tried the carbohydrates used have little or no capacity for engendering the production of precipitating antibodies, although this does not rule out the presence of such slight activity as would be necessary to give positive results in more delicate tests; *e.g.*, the production of active immunity to infections, or skin reactions in human beings following

[4] *Cf.* Zozaya, J., *J. Exp. Med.*, 1932, **55**, 346.

intracutaneous injections. In this connection mention should be made of the experiments of Tillett and Francis (22), Schiemann and his collaborators (23), Enders (24), Sabin (25), Wadsworth and Brown (26), Ward (27), Felton (28), and the work by Avery and Goebel (29) and Francis (30) on the immunizing properties of the polysaccharides of Pneumococcus Type I.

That an increase in the output of antibodies such as Gonzalez and Armangué and Zozaya[5] have reported may be obtained by the use of adsorbents is supported by the work of Glenny on alum-toxin mixtures. Further evidence is afforded by the heteroalbumose experiment described, in which the immunizing effect was enhanced considerably since a preparation of heteroalbumose with which it was rather difficult to produce antisera gave more active sera when adsorbed to alum or charcoal.

For the immunization effects following adsorption to inorganic colloids several explanations may be considered. The adsorbent may serve as a vehicle by means of which the substances are taken up by cells or it may slow down their elimination or bring about a stronger inflammatory reaction. In support of the latter is the fact that the peritoneal cavity is markedly inflamed following the injection of the usual amount of kaolin (0.2 gm.). Since Forssman extracts are not always activated by adsorption to collodion particles, it would appear that to administer the material in the form of particles does not necessarily suffice to increase antigenicity. In the case of immunization effects obtained with foreign sera (and not with homologous serum), the antigenic property of the serum would still seem to play the decisive rôle.

One may infer that the effects observed following adsorptions such as those of Gonzalez and Armangué, and of Zozaya in so far as they can be reproduced, are also due to an increase of preexistent antigenic activity which might be attributed either to an antigenic function of the haptens themselves (31) or else to the concurrent action of other substances either mixed or combined with the haptens.

[5] In a later paper (*J. Exp. Med.*, 1933, **57**, 38) Zozaya tentatively explained marked differences observed in the antigenicity of various polysaccharides on the assumption that the carbohydrates had lost their immunizing property in the process of purification while retaining their serological activity.

SUMMARY

Experiments are described which confirm the observation of Gonzalez and Armangué that heterogenetic extracts can be made antigenic by adsorption to inorganic materials. With fractions of the original extracts from which a part of inactive material had been removed no such enhancement was observed, whereas with foreign protein an activation was still possible. Carbohydrate preparations behaved similarly in that purification, perhaps loss of protein, was accompanied by a distinct decrease in antigenicity after adsorption. The activity of a but slightly antigenic heteroalbumose preparation was markedly increased after adsorption to charcoal and alum. The most reasonable explanation for the effects observed by Gonzalez and Armangué, and Zozaya, seems to be that a preexisting antigenic capacity has been enhanced by the use of adsorbents. The experiments reported here support the view that these effects are influenced significantly by the presence of substances other than those of a specific nature.

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VIABLE LEISHMANIA DONOVANI IN NASAL AND ORAL SECRETIONS OF PATIENTS WITH KALA-AZAR AND THE BEARING OF THIS FINDING ON THE TRANSMISSION OF THE DISEASE

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Kala-azar is endemic in large areas of China, India and the Mediterranean basin. The most important unsolved problem in this disease is to find its natural mode or modes of transmission. This subject has been studied for 30 years by numerous workers both independently and as members of various kala-azar commissions. Much useful information has been accumulated, but no solution to the problem has been found.

The most favored theory is that the disease is transmitted by the bite of an insect. Numerous insects have been suspected, among them bed bugs, lice, ticks, mosquitoes and sand-flies. Of these the sand-fly of the genus *Phlebotomus* seems best to fulfil the requisites as an intermediate host. This insect readily becomes infected when feeding on cultures of *Leishmania donovani* or on patients or animals suffering from kala-azar; the parasites multiply and develop into flagellates in the intestine of the fly, and eventually there may occur massive infection of the fore gut and pharynx of the phlebotomus. There exist many other facts which suggest that the sand-fly is the transmitting agent of the disease but the practical experience of many workers using hundreds of thousands of sand-flies, many thousands of susceptible animals and not a few human volunteers has failed to confirm this theory of transmission. In only one animal has kala-azar been thought to have been transmitted by the bite of the sand-fly and in this one instance the evidence is not conclusive that the disease was actually transmitted by the bite of the insect (1).

The next most favored theory is that of direct infection through contact with infective material. It has been shown that both the urine and feces of patients suffering from kala-azar may at times contain viable leishmania. This mode of contaminative infection has been investigated by Shortt and his associates. They failed to infect any of thirty-two highly susceptible animals (Chinese hamsters)

by feeding them repeatedly over a long period of time on the deposit from the centrifuged urine of untreated cases of active kala-azar (2). They succeeded in infecting only one of thirty-two hamsters by feeding each animal repeatedly, over a long period of study, with the feces of hamsters and of patients suffering from kala-azar (3). These studies suggest that it is possible although not probable that the disease may be transmitted by contamination with the urine and feces of certain infected individuals without reference to the presence of an intermediate host. There exist no data in the medical literature on kala-azar concerning studies of other external excretions or secretions of the body.

It is common knowledge that the Leishman-Donovan bodies in an individual suffering from kala-azar exist in many organs of the body, most abundantly in the mesenchymal macrophages (clasmatocytes, reticulo-endothelial cells, histiocytes) which are found in the adventitial coat of blood vessels, in the common connective tissues, and most abundantly in the spleen, bone marrow, liver and lymph nodes. The parasites may at times be found free in the tissues or may be engulfed by polymorphonuclear or mononuclear leucocytes of the blood. Numerous investigators have shown that a few parasites can be found in the leucocytes of the blood in a large proportion of patients.

These facts, the known presence of leishmania in lymph nodes and in blood cells, suggested to us a new approach to the problem of the transmission of kala-azar. It is a well known fact that the disappearance of leucocytes from the blood stream is accomplished, at least to a considerable degree, by the pouring out of these cells into the gastrointestinal tract and onto the surfaces of mucous membranes. It is also known that certain structures, the tonsils and adenoids, in the upper alimentary and respiratory tracts are lymphoid organs, lymph nodes possessing a more or less specialized function by virtue of their anatomical position.

It is an interesting fact, indeed an almost unbelievable one, that the nasal and pharyngeal passages have not been searched for the presence of leishmania. There exist no records in the medical literature on kala-azar of the examination of the tonsils or adenoids or of the exudate from these organs, for the presence of leishmania. The reason for the lack of interest in this phase of the subject becomes apparent as one reads the papers of and talks with the workers in this field. There have existed certain axioms regarding the parasite and its transmission which have militated against the theory that the parasites,

TABLE I

Data concerning Patients with Kala-Azar in Whom the Nasal Contents Were Examined for Presence of Leishmanias

Case No.	Age	Sex	Duration of symptoms	Edge of spleen below left costal margin*	State of the blood			Leishman-Donovan bodies in smears from		Globulin test on blood	Remarks
					R.B.C. per c.mm.	Hemoglobin per 100 cc.	W.B.C. per c.mm.	Spleen or liver	Nose		
	yrs.		mos.	cm.	millions	gm.					
194	24	M	1.5	11.0	3.71	10.0	4,850	Moderate number	Pos.	++++	
195	26	M	1.5	9.0	3.05	9.2	2,500	Moderate number	Neg.	++++	
196	61	F	2	11.0	2.40	7.6	1,350	Moderate number	Neg.	+++	
197	11	M	2	6.0	2.24	7.8	1,500	Many	Pos.	+++++	
198	39	M	1	11.0	4.03	8.2	2,200	Few	Neg.	+++++	
P78	20	M	15	23.0	3.95	8.8	2,100	Many	Pos.	+++	Death. Autopsy. Many leishmania in tonsil
184	8	M	10	4.5	4.15	9.4	4,950	Few	Neg.	+++	
199	20	M	3	8.0	3.24	7.4	9,100	Moderate number	Pos.	+++	Noma. Death. No autopsy
187	8	F	7	16.5	2.77	7.5	3,500	Few	Pos.	+++	
189	4	F	17	16.5	3.58	7.9	4,400	Moderate number	Neg.	+++++	Recurrence after treatment
P102	38	M	12	7.0	2.22	6.8	4,500	Many	Pos.	+++	
P104	4	M	6	6.5	3.50	9.5	2,800	Moderate number	Pos.	+	
P105	14	M	10.5	22.0	2.40	6.0	1,800	Many	Pos.	+++	
P107	26	M	1.1	10.0	2.84	8.7	2,260	Few	Neg.	+++++	
P108	27	M	18	17.0	3.10	9.8	1,900	Many	Pos.	++	

*These measurements were from the mid-clavicular line at the left costal margin to the tip of the spleen.

particularly viable ones, could exist in the secretions or on the surfaces of mucous membranes.   Because the parasite is in one of the stages of its life cycle a flagellate, and is closely related to the parasite of African trypanosomiasis, known to be transmitted by the tsetse fly, it has been assumed by many workers, notwithstanding some evidence to the contrary, that an insect intermediate host is essential for transmission.   Another general conception, and in our opinion a false one, is that the parasite of kala-azar is a delicate organism not capable of surviving in the presence of bacilli and cocci and unable to resist much change in its chemical or physical environment.

<div align="center">EXPERIMENTAL</div>

During the last 5 months we have examined material obtained by gently passing ordinary culture swabs into the nasal passages of fifteen patients suffering from proved kala-azar and then smearing slides with the material so obtained on the swab.   In nine of these fifteen cases we have found in the smears typical Leishman-Donovan bodies in small numbers.   Some of the preparations revealed the leishmania readily after a few minutes of search whereas others required very careful study over periods of from 20 minutes to 2 hours before organisms were demonstrated.   The parasites were in all respects identical with those obtained by means of puncture of the spleen or liver of the patients, which procedure was carried out in each case.   The Leishman-Donovan bodies usually were extracellular although occasional ones were demonstrated within polymorphonuclear neutrophiles.   The parasites existed in the presence of many contaminating bacilli, cocci and in some instances spirochetes.   The Leishman-Donovan bodies were present also in the ordinary mucus or semipurulent material blown from the nose by the patients and existed independent of any bleeding or demonstrable mucous membrane lesions.   Table I gives some of the data concerning the fifteen patients examined.

In one instance (Case P78) a swab lightly passed over the surface of the tonsil and then onto a slide demonstrated that the surface of the tonsil was the residence of significant numbers of typical Leishman-Donovan bodies.   The saliva of this patient also contained a few leishmania.   The patient received 2.3 gm. of neostibosan in a period of 29 days but died of complicating pyogenic infection.   At autopsy the

tonsils were examined. Smears from them revealed massive infection. In some areas the smears contained many hundreds of leishmania in each oil immersion field. The section of the tonsil confirmed the impression gained from the smear. Many macrophages laden with parasites were in and under the mucous membrane. A small ulcerated area of the mucous membrane contained innumerable bacteria in polymorphonuclear cells and macrophages and in addition many leishmania both free and intracellular were in the exudate of the ulcer. Many parasites were scattered throughout the whole tonsil chiefly concentrated in macrophages in the lymph cords and in the germinal centers of the follicles.

Having proved that the parasites were commonly present in the nasal and oral secretions the next step in our investigation was to ascertain whether or not these leishmania were viable. The proof of this point in two cases was accomplished by injecting intraperitoneally into hamsters emulsions, in normal salt solution, of the exudate blown by the patients from the nose into sterile Petri dishes.

The hamsters were followed and, at the time of death or when sacrificed, smears were made from the spleen, liver and in some instances from lymph nodes. These smears were subsequently stained with Wright's stain and examined microscopically. Pieces of liver, spleen, lymph nodes, bone marrow, lung, kidney and occasionally of other organs were fixed in Zenker-formol (formalin 10 per cent) and after sectioning, stained with hematoxylin and eosin. The results of these studies are shown in Table II.

For the first case (No. P94) twelve normal hamsters of one lot were selected. Five of the animals were inoculated with 1.0 cc. of the emulsion. Seven were held as control animals. Of the five injected animals two died 14 days after injection and at autopsy showed no demonstrable cause of death and no leishmania in smears or sections of the spleen, liver and lymph nodes. These two animals did not live long enough for the experiment to be satisfactory. The remaining three injected animals were sacrificed at the end of 79, 93 and 93 days respectively. In two of these animals the spleen was slightly enlarged. Other than this no gross abnormalities were found. Two of the three inoculated animals which lived long enough for a satisfactory experiment showed in the smears and in sections of the liver and spleen at autopsy numerous leishmania. In one of these animals the peripheral lymph nodes were also infected, whereas the lymph nodes of the other animal were not examined.

Four of the seven control animals died spontaneously 10, 19, 21 and 21 days respectively after the beginning of the experiment. In one of these postmortem changes were marked and no tissues were examined. The tissues of the other three

TABLE II

Data concerning Intraperitoneal Inoculations into Chinese Hamsters of Emulsions in Normal Saline of Nasal Discharge from Two Patients with Kala-Azar

Case No.	Animal No.	Inoculated or control	Amount of emulsion injected (cc.)	Duration of experiment (days)	History of animal	Presence or absence of leishmania in smears of — Spleen	Liver	Lymph nodes	Presence or absence of leishmania in sections of — Spleen	Liver	Lymph nodes
P94	A21-H1	Inoculated	1	14	Found dead	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	A22-H2	Inoculated	1	14	Found dead	Neg.	Neg.	—	Neg.	Neg.	—
	A23-H3	Control	None	19	Found dead	—	—	—	Neg.	Neg.	—
	A24-H4	Control	None	21	Found dead	Neg.	Neg.	—	Neg.	Neg.	Neg.
	A25-H5	Control	None	21	Found dead	—	—	—	Pos.	Pos.	Pos.
	A26-H6	Inoculated	1	79	Killed	Pos.	Pos.	—	Neg.	Neg.	—
	A27-H7	Control	None	82	Killed	Neg.	Neg.	Pos.	Pos.	Pos.	—
	A28-H8	Inoculated	1	93	Killed	Pos.	Pos.	—	Neg.	Neg.	—
	A29-H9	Inoculated	1	93	Killed	Neg.	Neg.	—	Neg.	Neg.	Neg.
	A30-H10	Control	None	93	Killed	Neg.	Neg.	—	Neg.	Neg.	Neg.
	A31-H11	Control	None	93	Killed	Neg.	Neg.	—	Neg.	Neg.	Neg.
	A32-H12	Control	None	10	Found dead	—	—	—	—	—	—
P78	A33-H13	Inoculated	1	1	Found dead	—	—	—	—	—	—
	A34-H14	Inoculated	1	45	Killed	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.
	A35-H15	Inoculated	1	Incomplete	Living	Incomplete	Incomplete		Incomplete	Incomplete	
	A36-H16	Inoculated	1	Incomplete	Living	Incomplete	Incomplete		Incomplete	Incomplete	
	A37-H17	Inoculated	1	Incomplete	Living	Incomplete	Incomplete		Incomplete	Incomplete	
	A38-H18	Control	None	48	Killed	Neg.	Neg.	Neg.	Neg.	Neg.	—
	A39-H19	Control	None	Incomplete	Living	Incomplete	Incomplete		Incomplete	Incomplete	
	A40-H20	Control	None	Incomplete	Living	Incomplete	Incomplete		Incomplete	Incomplete	
	A41-H21	Control	None	Incomplete	Living	Incomplete	Incomplete		Incomplete	Incomplete	

showed no leishmania when either smears or sections were examined. The three remaining control animals were sacrificed at the end of 82, 93 and 93 days respectively and no leishmania were demonstrable in the tissues.

For the second case (No. P78) nine normal hamsters of the same lot were divided into two groups, one of five which were inoculated and one of four held as controls. The five inoculated animals each received 1 cc. of emulsion of the nasal discharge of the second patient. Of these five, one died within 24 hours after inoculation. At autopsy diffuse peritonitis was found. The tissues were not examined microscopically. One of the remaining four inoculated animals was killed 45 days after the beginning of the experiment. Smears of tissue from the spleen and liver showed no leishmania but the peripheral lymph nodes contained numerous typical leishmania. The remaining three inoculated animals are still living and will be examined later at a more satisfactory time for terminating the experiment. One of the four control animals was killed 48 days after the beginning of the experiment but no leishmania could be found in smears of the spleen, liver or lymph nodes. The remaining control animals are still living.

The results of our investigations have proved for the first time that leishmania can be demonstrated in the secretions of the nose or oral cavity or both in a large proportion of patients suffering from kala-azar, and that in two such patients, the only ones in which studies are complete, these leishmania were viable and capable of producing infection in susceptible animals.

The literature on experimental kala-azar contains numerous reports of the disease having been experimentally produced in hamsters, monkeys, mice, dogs and other animals by feeding with the infected tissues of man or animals or with feeding of cultures of the parasite. Indeed in one instance in man infection was believed to have been produced by the accidental sucking into the mouth of infected material (4).

There exists, therefore, a series of facts which strongly support the theory of the transmission of the disease from person to person by way of the upper respiratory and alimentary tracts. This evidence consists of the facts enumerated above and is recapitulated as follows: (1) A rich source of infective material is present in the discharges from the nose and mouth of patients suffering from kala-azar. (2) Animals and presumably man can be infected readily by the ingestion of infective material.

The only point which now needs to be demonstrated to confirm the finding that one of the natural modes, possibly the most important

natural mode, for the transmission of kala-azar from person to person, by way of the upper respiratory and alimentary tracts, is actually to transmit the disease from an infected to a normal individual by these routes.

This experiment has been approached in two ways, by transferring infected material from the nasal cavities of patients to the nasal and mouth cavities of hamsters and to the nasal cavities of two human volunteers. It is still too early to report the results of these experiments.

The concept of the transmission of kala-azar from man to man by way of the upper respiratory and alimentary tracts suffices to explain many of the epidemiological problems in the disease. There is no serious objection to this concept either from the protozoological or the epidemiological point of view. A thorough consideration of these aspects of the problem will be given elsewhere.

SUMMARY AND CONCLUSIONS

1. Smears from the nasal cavities of fifteen patients suffering from kala-azar have been examined and in nine of these typical Leishman-Donovan bodies have been found.

2. Smears from the surface of the tonsil and from the saliva in one of the above nine cases showed the presence of leishmania. The tonsils of this patient, who died as the result of kala-azar and secondary infection, at autopsy were shown to be massively infected with Leishman-Donovan bodies.

3. Leishmania in the nasal discharge of two patients were shown by inoculation into susceptible animals to be viable and capable of producing infection. Sufficient time has not elapsed to determine the viability of the organisms from the remaining cases.

4. These experiments show for the first time that a rich source of infective material from a large proportion of patients with kala-azar is available for direct transmission of the disease.

5. Strong evidence is presented as a basis for the concept that one of the natural modes, perhaps the most important natural mode, of transmission of kala-azar is from person to person by way of the upper respiratory and alimentary tracts.

6. Two normal human volunteers and numerous normal experimen-

tal animals have been inoculated into the nasal and oral cavities with the nasal discharge, known to contain leishmania, from patients with kala-azar. The results of these experiments will be reported at a subsequent date.

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LYMPHOMATOSIS, MYELOMATOSIS, AND ENDOTHELIOMA OF CHICKENS CAUSED BY A FILTERABLE AGENT*

II. MORPHOLOGICAL CHARACTERISTICS OF THE ENDOTHELIOMATA CAUSED BY THIS AGENT

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PLATES 31 TO 35

Begg (1) and Murray and Begg (2), the first to describe endothelioma in chickens, proved that it was caused by a filterable agent. In a previous article (3) we have reported on a transmissible strain of leukosis of fowls (Strain 2) caused by a filterable agent that stimulates to apparently neoplastic growth not only several types of blood cells, but also endothelium.

All filterable agents of chicken sarcoma thus far described are specific in the sense that they produce morphologically characteristic lesions (Claude and Murphy (4)), and the same was found by us to be true for two strains of chicken leukosis. Oberling and Guerin, on the other hand, suppose that the virus observed by them recently (5) was at first the common virus of leukosis of Ellermann and that this virus, while under their observation, mutated into a virus with affinity for mesodermal and ectodermal tissues.

Neoplastic processes are common in chickens and it is erroneous to attribute to the inoculum all types of neoplastic conditions occurring among passages of a virus. Our recent experience with Strain 2 is noteworthy in this connection. While making passages with it two new transmissible sarcomas were isolated. One, a sarcoma composed of spindle and polymorphous cells and abundant collagenous fibers, occurred among the uninjected controls. Its subpassages were free from leukosis. Another, an osteochondrosarcoma, occurred in a chicken injected with Strain 2, and among the birds inoculated with tissues of this tumor there occurred leukosis, osteochondrosarcoma, and leukosis mixed with osteochondrosarcoma.

Filterable agents of tumors and of leukosis present great variations in the location and morphological appearance of the lesions they produce.   For instance, our Strain 2 is capable of producing a variety of anatomically different lesions such as endothelioma, lymphomatosis, myelomatosis, and erythroleukosis.   Moreover, each of these lesions occurs in a variety of forms, and the type of disease produced is independent of that of the donor.   It is obvious that considerable work is required before the morphological range of a transmissible strain is established; and the occurrence of two instances of non-metastasizing epithelial growth, as observed by Oberling and Guerin among chickens inoculated with their leukosis virus, is insufficient evidence for the assumption that they were caused by the agent of leukosis.

In this article the appearance of endothelial growth as it occurs among passages of Strain 2 will be described, and in the article that follows the results of experimental work aiming to determine the relation of endothelioma to leukosis will be described.   Endothelioma, infrequent as a spontaneous disease, appeared repeatedly in a variety of forms.   The relationship of these varieties of endothelioma was established by the observation of transitional forms among them, the occurrence of different lesions in the same bird and in different birds that had been injected with material of the same origin.

Nomenclature

Leukosis of fowls may be subdivided into: (1) lymphomatosis, (2) myelomatosis, and (3) erythroleukosis.   Lymphomatosis of Ellermann (6) is identical with hemocytoblastic myelosis of Battaglia and Leinati (7).   The origin and potentialities of the cells in the disease named by Ellermann lymphoid leukosis and by Battaglia and Leinati hemocytoblastic myelosis still remain to be determined.   Battaglia and Leinati consider them mother cells of granulocytes and erythroblasts; that is, hemocytoblasts in the sense of Ferrata (8).   The large basophile lymphocytes of Strain 2 seem to be able to produce erythroblasts, myelocytes, and lymphocytes, and therefore function as hemocytoblasts in the sense of Maximow (9).   For this reason we have decided to use the term hemocytoblasts as a synonym for the large lymphocytes of Strain 2.   Lymphomatosis or lymphoid leukosis of Ellermann includes a group of different diseases in some of which the predominating cells are well differentiated lymphocytes, as in neurolymphomatosis, and in others the predominating cells are cells like large lymphocytes, the potentialities of which are unknown.   No attempt will be made to subdivide lymphomatosis until our study of this group of diseases has been completed.   Myelocytomatosis is the term used for the disease in which the infiltrations or tumor masses are composed of myelocytes and of almost no other cells.   The term endothelioma refers to neoplasms of endothelium including those of the capillaries of the liver, spleen,

and bone marrow. Often it may be impossible to determine whether the tumor cells are of endothelial, mesenchymal, or mesothelial origin. Similar difficulties have been met with in tissue culture studies of these cells, and Fischer states (10)[1] that the endothelial cell of one investigator may correspond to the fibroblast of another investigator.

Material of Study. Causation of Endothelioma. Associated Leukotic Changes

The origin and character of Strain 2 and methods of its transfer have been described (3).

Among the first 150 chickens successfully inoculated with this strain 11 instances of leukosis were associated with endothelioma with giant cells. The 150 instances of leukosis were classified as follows: lymphomatosis (hemocytoblastosis) 72, myelocytomatosis 17, myeloblastomatosis 2, erythroleukosis 8, atypical (mixed) leukosis 38, and untyped 13 (3). 3 instances of endothelial neoplasm unassociated with giant cell formation found in the same series and 17 instances of endothelial neoplasms found among chickens inoculated more recently with Strain 2 are the subject of this study.

Table I shows some significant data on 33 chickens with endothelioma caused by Strain 2, and surveyed in this paper.

The weight of the chickens varied at the time of inoculation from 110 to 1300 gm. All chickens studied were baby chicks or young adult Barred Rock chickens. Four received sublethal doses of x-rays before inoculation, the rest were not irradiated.

All instances of endothelioma observed were associated with either myelocytomatosis or hemocytoblastosis, or both. Since four cases were produced with cell-free agents (dried blood, plasma, plasma filtrate, and frozen blood respectively) and since all but three of the other cases were caused by inoculation of fresh blood, it is evident that endothelioma arises through stimulation of endothelial cells of the host with no implantation of tumor cells. All but four of the chickens whose blood was used for transfers were free from endothelioma, and it is unlikely that even in these four endothelial cells were present in the circulating blood. It is likewise improbable that the large lymphocytes circulating in the blood of chickens inoculated with this

[1] Fischer (10), p. 391.

TABLE I

Data on Chickens with Endothelioma Caused by Strain 2

Chicken		Inoculum			No. of passage	Type of disease in donor	Length of life after inoculation	Blood changes		Type of disease
No.	Weight	Material	Amount	Route				Duration	Morphological	
	gm.		cc.				days	days		
2843	900	Blood	1	iv.	II	Atypical	90	47	an, mc, h	sMc, Enh
2847	110	"	0.5	"	II	"	88	38	h, mc, an	sMc, Eng
3146	420	"	0.005	"	II	lMc	92	41	h, an	sL, Eng
3150	490	Plasma	1	"	II	lMc	36	18	er, h	aMc, Eng
3198	—	Blood	1	"	II	Atypical	55	16	er, h, mc	Atypical, En
2989	800	"	6	"	III	sL	67	18	h, an	Eng, sL
2997	900	"	0.5	"	III	sL	64	46	h	sL, En
3302	1140	"	1	"	III	sL	118	7	an, mc	sMc, Eng
3227	810	"	1	"	IV	sL	39	23	er, h, mc	Mc, Eng
3236	1020	"	0.005	"	IV	sL	88	—	Negative	sMc, Eng
3241	1140	"	1	"	IV	Atypical	45	30	an, h, mc	Atypical, Mc, Eng
3113	1200	"	1	"	V	"	51	29	an, h	" Eng
3307	1120	"	1	"	V	" Eng	51	36	h, an, later negative	" Eng
3349	300	"	1	"	V	"	69	22	h, an, mc	" Eng
3432	980	"	3	"	V	sMc	56	29	h	" En
3443	1170	Frozen cells	0.005	"	V	sL	48	26	an, h, mc	" Enh
3444	1300	Blood	0.005	"	V	sL	63	43	er, h, mc, later negative	" Enh
3488	200	Blood cells	—	"	V	sL	32	—	an, h, mc	sL, Eng
3489	180	" " "	—	"	V	sL	63	4	h, an, mc	Atypical, Eng
3491	210	" " "	—	"	V	sL	43	23	an, h	sL, En

		Dried cells	0.1							
35.16	200	Blood	0.2	iv.	V	sL	43	24	an, h	Atypical, mainly Mc, Eng
3334*	2.10	"	0.2	"	VI	smc, Eng	68	18	an, mc, h	Atypical, Enh
33.13	200	"	0.5	"	VI	Atypical, Eng	64	—	an	aL, Enh
3366*	320	"	0.5	"	VI	1L	39	13	er, h, mc	Atypical, Enh
3372*	2.10	Plasma fil-trate	4.5	"	VI	1L	62	19	an, h, mc	sL, Enh
3396	230	Blood	5	"	VI	1L	90	43	an, h	Atypical, Ensa
3406	890	"	2	"	VI	Atypical	36	4	h, an	1L, Eng
3572	750	{ Tumor	—	ith.	VI	"	56	26	an, h	Atypical, mainly Eng, Enh
3338*	200	Blood	0.2	iv.	VII	sMc, Eng	36	25	an, h	Atypical, Eng
3437	8.10	Tumor	—	im., ith.	VII	sL	42	14	h, mc	sL, Eng
3167	1190	Washed blood cells	—	iv.	VII	Atypical	35	15	er, h	Atypical, Enh
3500	220	Tumor	—	im.	VII	"	62	—	h, mc	1Mc, Eng
3.180	1000	Blood	3	iv.	VIII	1L	40	25	an, h	sL, Eng

* These chickens were irradiated before inoculation with sublethal doses of x-rays.

Abbreviations Used in Table I

Type of Leukosis.—L = lymphomatosis (hemocytoblastosis), Mc = myelocytomatosis, E = erythroleukosis, Eng = endothelioma with giant cell formation, Enh = endothelioma with hematoma, Ensa = endothelioma with sarcoma-like growth, En = all other forms of endothelioma. Instances of leukosis in which the involvement of two or more systems was conspicuous are given as atypical leukosis.

Degree of Blood Involvement.—(The blood involvement is described by an adjective used with the abbreviation for the type of leukosis.) l = leukemic, s = subleukemic, a = aleukemic.

Morphological Blood Changes.—(The changes are those seen in blood smears.) an = anemia, er = anemia suggestive of erythroleukosis, mc = myelocytes, h = hemocytoblasts (large basophile lymphocytes).

Route of Injection.—iv. = intravenous, ip. = intraperitoneal, im. = intramuscular, ith. = intrathymic.

strain would have changed into endothelium. Moreover, morphological appearances indicated that endothelioma developed *in situ* through proliferation of preformed endothelium.

The blood smears of all but one chicken gave evidence of leukotic changes. The changes were first observed from 11 to 59 days after injection and lasted until death in all but two chickens. In these two the immature cells disappeared from the peripheral blood several days before death. In most cases the presence of numerous polychrome erythrocytes and erythroblasts and occasional basophile erythroblasts in the blood indicated anemia, and in a few cases the relatively large number of basophile erythroblasts suggested erythroleukosis. The presence of large numbers of cells like lymphocytes in the bone marrow, presumably mostly primitive erythroblasts, also often suggested erythroleukosis, but stasis of these cells in internal organs, the most characteristic feature of erythroleukosis, was not observed in the chickens in which endothelioma was associated with leukosis. Large basophile lymphocytes (hemocytoblasts), illustrated in our previous communication (3),[2] were seen in the blood of all but three chickens; their number varied from a few to approximately 100,000 per c.mm. Thus there were no blood changes characteristic of endothelioma; the blood smears showed the presence of either secondary anemia, or erythroleukosis; in others, myelocytomatosis, or hemocytoblastosis, or, most commonly, mixed leukosis (Table I).

The birds died from 32 to 118 days after inoculation. Some were emaciated at the time of death; others were well nourished and died suddenly of hemorrhage from an endothelial tumor.

Morphological Characteristics

In many instances the endothelial growth was small and was discovered only on microscopic examination. In the majority of instances, however, stimulation of endothelium resulted in the formation of tumors varying greatly in size, from a few millimeters to 7 cm. One large tumor attached to the liver is shown in Fig. 1.

Endothelial growth varied in color; it was yellowish grey, grey, or red, and it was often spotted by minute, yellowish, necrotic areas. In contrast to endothelioma, myelocytoma nodules were white, and lymphomatous tumors grey. Necrosis and hemorrhage were common in endothelioma.

The small yellowish areas with endothelioma were composed of necrotic tissue and were surrounded by multinuclear giant cells (Fig. 9). These cells often showed in sections cut at 7 μ from twenty to

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[2] Furth (3), Figs. 3 to 5.

fifty nuclei, and it is estimated that many contained hundreds of nuclei (Fig. 6). They resembled those described and illustrated by Murray and Begg (2). Tumors with giant cells were found in eleven of the first 150 birds successfully inoculated with Strain 2, and their occurrence called our attention to the ability of Strain 2 to produce endothelioma. Other types of endothelial growth were not considered in our first report.

Since necrosis was often absent in endothelial tumors with giant cells, it is evident that the formation of the giant cells is not secondary to necrosis. A giant cell tumor free from necrosis is shown in Fig. 6 and a similar neoplasm invading the bone marrow is shown in Fig. 8. In Fig. 6 there are numerous transitional forms between mononuclear and multinuclear giant cells. This photomicrograph, taken of a tumor in the lumbar region, shows invasion of nerves. In sections of giant cell tumors with necrosis, stained by Ziehl-Neelsen's method, no microorganisms were seen.

The nuclei of the endothelial tumor cells and notably those of endothelial giant cells are, in contrast to those of most malignant cells, poor in chromatin. They contain a large, sharply outlined, spherical body stained intensely red with azure, that takes the place of the nucleolus but lacks its basophilia. Giant cells are usually considered as evidence of disturbed cell division; the nuclear characteristics of the endothelial giant cells may be regarded as morphological evidence of cell injury.

Endothelioma with giant cell formation was observed in the following locations: liver, lung, mesentery, intestinal tract, thymus, bone marrow, spleen, ovary, pancreas, skin, eye, kidney, and voluntary muscle. Since only a few tumors of each bird were examined microscopically, the distribution and frequency of this type of lesion cannot be accurately given. It seems to be most common in the liver. The following is the brief history of a fowl with endothelioma showing giant cells.

No. 3302.—The bird was inoculated Dec. 17, 1932, intravenously and intramuscularly with lymphomatous tumor tissue of No. 2930. Blood smears taken repeatedly until Mar. 20, 1933, were negative. On Apr. 4 a few polychrome erythrocytes and erythroblasts and myelocytes were seen in the blood smear. It died Apr. 5. The bird was well nourished and well developed. There was no

tumor at the site of inoculation. Attached to the breast bone there were numer-
ous whitish myelocytoma nodules. Endothelioma with numerous multinuclear
giant cells had caused consolidation of the greater part of the lung. The liver
was studded with minute white nodules of myelocytoma and contained an occa-
sional yellowish grey nodule of endothelioma with multinuclear giant cells (Fig. 9).
In the portal area mesenchymal or endothelial growth was associated with myelo-
cyte formation as shown in Fig. 18. The cavity of the bone marrow was narrowed
by spongy, in greater part osteoid, tissue, in the meshes of which were numerous
multinuclear giant cells (Fig. 8). The marrow of the femur was spotted thickly
with large necrotic areas surrounded by tumor tissue with giant cells. The pulp
of the spleen was congested and studded with very large mononuclear cells and
giant cells with from two to seven nuclei (Fig. 7). Germinal centers were absent;
in their place there were foci composed of large round and multinuclear cells like
those that occupied the pulp. These abnormal cells seemed to have originated in
the endothelium of the spleen. Their invasive property was made manifest by
their penetration through and destruction of the muscular wall of large veins.
There were many myelocytes originating in the spleen. In the heart muscle there
were small foci of extravascular, diffuse infiltrations composed of tumor cells like
those seen in the spleen. The tumors attached to the bones were composed largely
of myelocytes that were invading the surrounding fatty tissue. Adjacent to these
myelocytomas there were areas of necrosis with multinuclear giant cells.

The endothelial character of some of the tumor cells of Strain 2
was clearly shown when multiple endothelioma was associated with
hematoma, as illustrated in Fig. 3 (No. 3467).

In one chicken (No. 3443) bleeding from an endothelioma with hematoma,
approximately 0.5 cm. across and situated at the upper part of the right liver lobe,
was the immediate cause of death. In No. 3467 the hematomata were surrounded
by endothelial cells forming a coherent sarcoma-like growth. These cells sur-
rounded cavities or channels and formed irregular or papillomatous prominences
projecting into the lumen (Fig. 4). Within the cavities thus formed hemocyto-
blasts were abundant and mature erythrocytes were present in small numbers,
whereas in the adjacent hematoma erythrocytes were abundant and leukocytes
were few. The nuclei of most of the endothelial cells were swollen; many of these
cells protruded into the channels or cavities formed by the tumor cells and some of
them were detached (Fig. 4). Some of these detached large round cells with large
pale stained nucleus and dark stained nucleolus resembled closely the endothelial
cells; other cells of smaller size with darker stained nucleus with conspicuous
nuclear structure resembled hemocytoblasts, and there were transitional forms
between these two types of cells.

The following is the brief history of a fowl having endothelioma with
multiple hematomata.

No. 3467.—The bird was inoculated intravenously on Mar. 16, 1933, with washed blood cells of No. 3397. On Apr. 11 there were numerous polychrome and basophile erythroblasts in the blood smear, later myelocytes and basophile lymphocytes appeared in the blood and mitotic figures among the erythroblasts were numerous. It was killed for study on Apr. 20. The mesentery was studded with 1 to 4 mm. red tumor nodules (Fig. 3) formed by the endothelial growth just described. Similar tumor nodules were seen in the spleen (Fig. 3) and ovary. At the aperture of the thorax there was a hematoma of approximately 3 cm. across, the origin of which was not determined. In the liver, nerves, adrenal, and the adjacent sympathetic ganglion, there was mild to moderate infiltration by hemocytoblasts, and mitosis of these cells in the infiltrated tissues indicated multiplication *in situ.* The marrow was almost completely replaced by hemocytoblasts and erythroblasts.

Endothelioma with the formation of channels, but with little or no differentiation of endothelial cells into blood cells, is shown in Fig. 11 (No. 3572).

This section was taken from one of several small (3 to 5 mm.) red tumors that were attached to the rib. On inspection with low magnification the tissues composing the greater part of this tumor resemble young, loose, connective tissue. It is evident with higher magnification, however, that the endothelium of the sinusoidal capillaries of this tumor is hypertrophied and hyperplastic, and that many of the elongated cells in the loose tissue surrounding these capillaries are in appearance indistinguishable from the lining cells of the capillaries. This picture recalls experiments made with tissue culture (*cf.* 10) that showed transformation of endothelial cells into cells indistinguishable from fibroblasts, and it illustrates the difficulties of distinguishing young endothelial cells from young fibroblasts.

Similar but more compact sarcoma-like endothelial growth was seen in the bone marrow of this bird (Figs. 12 and 13). There were numerous areas of hematoma in this organ and the greater part of it was occupied by primitive cells like large lymphocytes. It showed little if any sign of endothelial proliferation. Amidst this hyperplastic marrow there were, however, areas with multinucleated endothelial giant cells. Such alterations show that the agent of Strain 2 has an affinity for both endothelium and primitive blood cells, and that stimulation of each type of cell occurs independently.

In the breast muscle there was a tumor approximately 1 cm. across. This tumor was red at the periphery, grey in the center. It was composed of nests of cells like large lymphocytes in endothelium-lined

spaces and separated from each other by dense connective tissue (Fig. 10).

Thus in this chicken two types of endothelial neoplasms were found; one was formed by sarcoma-like cells, the other by multi-nuclear giant cells.

Endothelial tumor growth of a different pattern, occurring in No. 3607, is shown in Figs. 15 and 16.

In the solid tumor there were numerous narrow clefts, each surrounded by several layers of hyperplastic endothelium and the lining endothelium was part of this growth. The adult connective tissue matrix that separated the foci of endothelial cells was abundant in some parts of the tumor, scarce in other parts. The endothelial tumor shown in these figures resembles the fibro-angio-endothe-lioma of Ewing (11).[3] Endothelial neoplasms, mostly with hematoma and varying in size from a few millimeters to about 3 cm. in longest diameter, were found in the liver, lung, thymus, kidney, ovary, mesentery, and muscles of this bird.

Some of the hematomata seen in this bird were evidently of old standing, for one in the liver and another in the kidney were surrounded by fibrous capsules of more than 100 μ in thickness, and in the kidney some of the fibroblasts of this thick capsule were growing into the hematoma.

The origin of some of the hematomata in endothelial growth was not shown by the sections made, but it is possible that this might have been shown through serial sections. Minute endothelial lesions detectable only by microscopic examination may obviously give rise to extensive hematoma. Such was the case in No. 3334, in which there was a hematoma 1.5 $\times$ 1.5 $\times$ 2 cm. at the aperture of the thorax. At the periphery of this hematoma there were incomplete tubules, mainly composed of cuboidal endothelial cells and containing eryth-rocytes (Fig. 14).

In different organs of the same bird the microscopic appearance of endothelial growth varied greatly; e.g., in this bird (No. 3334) there was also a solid tumor in the liver, about 1 cm. across, that showed, under the microscope, a sarcoma-like growth similar to that illustrated in Fig. 17.

Tubule formation resembling adenoma or adenocarcinoma, as shown in Fig. 5 (No. 3366), was seen in the liver of several chickens.

[3] Ewing (11), Fig. 132.

The endothelial character of the cells forming such tubules is indicated by their origin in endothelium, by the character of the nucleus of the cells, and by the presence of blood cells in the tubules. The continuity of the cuboidal cells forming these tubules with the endothelial cells of the capillaries of the liver is clearly seen at the edge of the neoplasm where part of the capillaries are formed by normal flat endothelial cells and part by cuboidal endothelial cells. Stimulated endothelium of liver capillaries assumed either gland-like growth as shown in Fig. 5, or grew in a densely packed, perhaps syncytial mass of tumor cells as shown in Fig. 17.

Growth of endothelial or mesenchymal cells was often found in the portal area of chickens inoculated with Strain 2. Fig. 18 (No. 3338) shows a dense mass of cells that resemble cytologically the endothelial cells already described. Adjacent to them are myelocytes and some of the myelocytes can be distinguished from the mesenchymal or endothelial cells merely by the presence of eosinophile granules. This suggests that the myelocytes have arisen by the deposition of granules in the non-granular endothelial or mesenchymal cells. Fig. 18 may be interpreted as morphological evidence of the transformation of mesenchyme or endothelium into myelocytes without the intermediary stage of hemocytoblast or myeloblast (Maximow (9)).[4] In another part of the liver of this bird (No. 3388) the endothelial or mesenchymal cells were elongated and one cell was anastomosed with another.

A section taken from the large tumor shown in Fig. 1 showed only sarcoma-like growth (Fig. 2). The endothelial character of this tumor is suggested by the formation of channels. Its causation by Strain 2 is suggested by the results of the transmission experiments because the two chickens injected with an emulsion of this tumor tissue died of lymphomatosis.

Sarcoma-like growth observed in two chickens after intramuscular transmission of Strain 2 will be more fully described in the article that follows. The growth appeared at the site of injection and distant lesions showed only leukotic alterations.

Ciaccio (12) was the first to describe syncytial tumor growth of endothelium in man, to which he gave the name of syncytial endothelioma. The microscopic appearance of this growth in the lymph node of a man bears, as shown by the photographs of Ciaccio, a striking resemblance to some of the growth caused by the agent of Strain 2. Ciaccio, too, was unable to decide whether the cells forming the tumor were endothelial or mesenchymal cells of the lymph node, but rightly considered that the histogenetical, anatomical, and functional relations of these

[4] Maximow (9), p. 372.

two types of cells are so close as to make it probable that the unknown agent that gave rise to the tumor might have stimulated either type of cell.

Diffuse infiltration by detached cells resembling large lymphocytes and by mesenchymal or endothelial cells forming dense, perhaps syncytial masses were not uncommon in the same bird and in the same organ. In some organs, such as nerves, the infiltrating cells were almost invariably detached. Occasionally, however, the cells infiltrating the nerves were large, palely stained, and appeared to be connected. Both types of infiltration occurring in the same nerve are shown in Figs. 21 and 22; the cells at the periphery of the nerve are the anastomosing cells and appear to have orginated either in perineural mesenchyme or in endothelium. The cells that infiltrated the central parts of the nerve resembled large lymphocytes.

A tumor composed of large, round cells (Figs. 19 and 20) (No. 3432) was found adjacent to the thyroid gland. It measured approximately 1 cm. across, contained thymic tissue, and apparently originated in or about a thymic lobe. The large size of the cells forming this tumor, their pale stained nucleus with compact dark stained nucleolus, and the numerous binucleated cells distinguish them from primitive blood cells. There was no evidence of maturation of these cells into blood cells. Similar tumor growth was seen in the mesentery of other chickens invading the proventriculus, gizzard, duodenum, and pancreas.

Evidence for the view that endothelium stimulated by the agent of Strain 2 produces blood cells is furnished in Fig. 23 (No. 3489). Here is seen the development of hemocytoblasts from endothelium within a sinusoid of the bone marrow lined by the activated or neoplastic endothelium. This sinusoid is surrounded by young, granular leukocytes. This picture, as well as Figs. 24 and 18 suggests, furthermore, that myelocytes may arise by the deposition of acidophile granules in endothelial or mesenchymal cells. By contrasting Fig. 23 with the figures shown in the report of Ratcliffe and Furth (13) on the pathogenesis of erythroleukosis, it can be observed that Strain 1 activates the primitive blood cells and not the endothelium of the marrow, whereas Strain 2 may activate both types of cells.

Sections are less suitable than blood smears for the study of the

maturation of hemocytoblasts into various types of blood cells. Blood smears of chickens inoculated with Strain 2 show abundant transitional forms between hemocytoblasts, on the one hand, and typical erythroblasts, myelocytes, and lymphocytes, on the other hand.

DISCUSSION

Type of Cells Stimulated by Strain 2. Hemopoietic Potentialities of Mesenchyme, Endothelium, and Primitive Blood Cells.—Sufficient data are not available to present a clear-cut picture of the pathogenesis of Strain 2, which seems to cover almost the entire field of blood formation. It causes a tumor-like multiplication of mesenchyme or endothelium. These adherent, or perhaps syncytial, cells either remain undifferentiated or they become detached and transformed into cells like large lymphocytes (hemocytoblasts). In blood smears of chickens injected with Strain 2 there are numerous transitional forms between these large lymphocytes, on the one hand, and erythroblasts, myelocytes, and small lymphocytes, on the other hand. Myelocytes appear also to arise in cells of endothelial type without the intermediary stage of large lymphocytes. Most myelocytes develop, however, by mitotic division of myelocytes, and most erythroblasts develop by multiplication of primitive erythroblasts.

The endothelial, mesenchymal, or mesothelial origin of the growth often cannot be determined (*cf.* the studies on the morphological and functional relationship of endothelium to mesenchyme (9, 10, 14)). It is evident, however, that several genetically closely related types of cells may become stimulated by the agent of Strain 2.

Sabin, Doan, and Cunningham bring evidence to show (*cf.* 15) that lymphocytes and granulocytes arise from mesenchyme, erythroblasts from endothelium. Since endothelium of the blood-forming organs, according to the now prevailing view, is flattened mesenchyme, the distinction between the views of these investigators and those who believe that all blood cells are derived from a single type of fixed cell has little significance. Our observations are in accord with those of Sabin, Doan, and Cunningham and indicate that erythroblasts arise intravascularly in the chicken, myelocytes and lymphocytes extravascularly, usually perivascularly. The mother cell of all blood cells is obviously either mesenchyme or its differentiated form, the endo-

thelium of the blood-forming organs, and the type of cell produced depends upon the growth stimulus. Under ordinary conditions the endothelium of the blood-forming organs of adult chickens is not hemopoietic, and blood cells arise mainly by multiplication of primitive blood cells. Nevertheless, when stimulated by agents such as that of Strain 2, the endothelium may become hemopoietic. It is noteworthy that in the chick embryo the formation from endothelium of large round cells like hemocytoblasts may be observed in numerous organs. Nonidez (16) has described it in the ovary, testis, meso- and metanephros, adrenal, and epididymis.

Local conditions influence the type of growth produced by the agent of Strain 2. Certain organs or parts of organs have a predisposition for characteristic growth; e.g., the cells infiltrating the nerves and myocardium are almost exclusively large lymphocytes, the tumors attached to the bones are composed of myelocytes, and of almost no other cells, and the cells formed within the sinusoidal capillaries of the marrow are largely primitive erythroblasts. The tumors of the mesentery are usually composed of very large round cells or giant cells. The growths induced in the breast muscle and thymus by injections of tissues containing the agent of Strain 2 are usually hemocytoblastic, and occasionally hemocytoblastic and sarcoma-like.

In order to obtain conclusive evidence of the origin of growths produced by the filterable agents causing tumors and leukosis as also of the potentialities of endothelium, mesenchyme, and primitive blood cells stimulated by these filterable agents, studies on living tissue (e.g. in culture, or in the Sandison-Clark chamber) are desirable.

Histological Types of Endothelioma in Man and in Fowl.—Most histological types of endothelioma described in man have been observed in chickens injected with Strain 2. Ewing's "interfascicular" type has been described here as sarcoma-like, his "alveolar" type as glandular, his "plexiform" and "diffuse" types as sarcoma-like or syncytial. Giant cells are rare in endothelioma of man, common in endothelioma of chickens. Brosch and Ewing describe giant cells of the Langhans type in endothelioma of man (cf. Ewing), and some of the multinuclear giant cells of avian endothelioma resemble closely those of an avian tubercle. Endothelioma of the bone (endothelial myeloma of Ewing) is rare in chickens but myeloma (myelocytoma) is common. Evidence has been presented above that the myelocytes of chickens may originate in mesenchymal or endothelial cells under the stimulus of the same filterable agent that causes endothelioma. The

similarity between syncytial endothelioma of man (*cf.* Ciaccio) and of chickens has already been mentioned. The association of endothelioma with leukemia is rare in man. Ewing observed a case of lymphatic leukemia associated with alveolar endothelioma. The tumors arising in endothelium of lymph nodes and usually classified as large-cell lymphosarcoma may be considered as a link between leukemia and endothelioma.

CONCLUSIONS

When stimulated by a filterable agent of leukosis of chickens (Strain 2), endothelium may undergo seemingly unrestricted growth. These neoplasms of endothelium are usually unaccompanied by the formation of blood cells. Occasionally they produce hemocytoblasts, discharged like those of the normal marrow into vascular channels as also myelocytes about the vessels.

The same agent that stimulates endothelium also stimulates erythroblasts, myelocytes, and hemocytoblasts to unrestricted growth without obviously affecting the endothelium; and the association of endothelioma and leukosis is the result of stimulation of several types of cells by a single virus. Myelocytes appear also to develop from mesenchymal or endothelial cells without the intermediary stage of hemocytoblasts.

It is often impossible to determine whether the neoplasms caused by the virus of Strain 2 are of endothelial or mesenchymal origin, and it is possible that both types of cells may be stimulated by the same virus. Types of sarcoma like those described by Rous are not produced by the virus of Strain 2.

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EXPLANATION OF PLATES

All sections were prepared from tissues fixed in Zenker-formol solution and were stained either with hematoxylin-eosin or with hematoxylin-eosin-azure solutions. The magnifications given are approximate.

PLATE 31

FIG. 1. (No. 3396.)   A large tumor (a) attached to the left lobe of the liver, a small tumor (b) in the upper part of this lobe, and two tumors (c) at the site of the ovary.

FIG. 2. (No. 3396.)   Microscopic appearance of the endothelial tumor of the liver shown in Fig. 1.   The tumor cells form rows and tubules.   × 100.

FIG. 3. (No. 3467.)   Multiple endotheliomata with hematomata of the liver.

FIG. 4. (No. 3467.)   Microscopic appearance of the small irregular cavities in the endothelial growth shown in Fig. 3.   The cavities are lined by endothelial cells and several of these cells are detached.   The cavities are filled chiefly with hemocytoblasts.   × 300.

PLATE 32

FIG. 5. (No. 3366.)   Endothelioma of the liver composed of cuboidal cells that are continuous with the endothelium of the liver capillaries.   × 300.

FIG. 6. (No. 3546.)   Endothelioma with giant cell formation infiltrating nerves.   × 100.

FIG. 7. (No. 3302.)   Diffuse endothelioma with giant cell formation in the spleen.   × 300.

FIG. 8. (No. 3302.)   Endothelioma with giant cell formation in the marrow cavity of the femur.   × 150.

FIG. 9. (No. 3302.)   Endothelioma with giant cell formation in the liver.   × 50.

PLATE 33

FIG. 10. (No. 3572.)   Microscopic appearance of a red tumor occurring in the muscle.   Abundant connective tissue separates nests of intravascularly located hemocytoblasts.   × 150.

Fig. 11. (No. 3572.) Microscopic appearance of a small endothelial tumor that was attached to the rib. The endothelium of the sinusoidal vessels is greatly hypertrophied and the elongated cells in the loose tissue surrounding the sinusoidal vessels are in appearance indistinguishable from the endothelial cells that line these channels. × 300.

Fig. 12. (No. 3572.) Shows two types of lesions in the bone marrow: (a) sarcoma-like growth of endothelial cells; (b) hyperplasia with formation of erythroblasts and cells like large lymphocytes. × 80.

Fig. 13. High magnification of the sarcoma-like growth of endothelial cells shown in Fig. 12. × 250.

PLATE 34

Fig. 14. (No. 3334.) Endothelial growth at the edge of a hematoma. × 250.

Figs. 15 and 16. (No. 3607.) Endothelial growth with the formation of narrow clefts surrounded by tumor cells. Abundant connective tissue separates the foci of endothelial growth. Magnifications: Fig. 15, × 100; Fig. 16, × 300.

Fig. 17. Extensive endothelial growth in the liver of No. 3490. × 300.

Fig. 18. Mesenchymal or endothelial growth in the portal area of the liver (No. 3338). There are numerous eosinophile granules in many of the tumor cells. × 300.
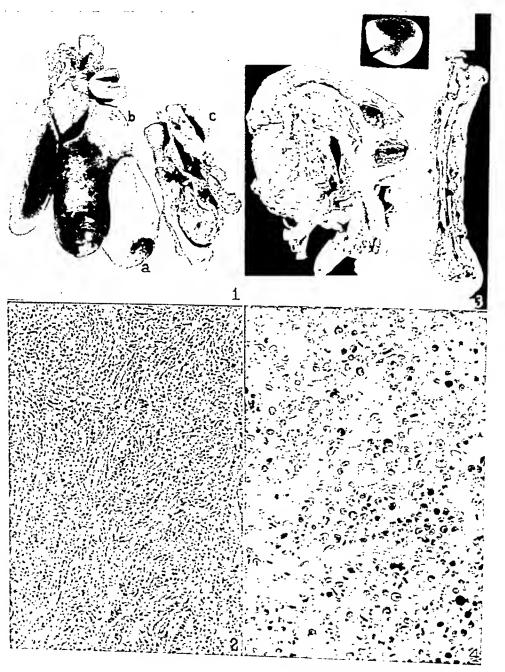
PLATE 35

Figs. 19 and 20. (No. 3432.) Large round cell sarcoma of the thymus, probably of endothelial origin. Magnifications: Fig. 19, × 150; Fig. 20, × 700.

Figs. 21 and 22. (No. 3571.) Two types of infiltration in nerves, hemocytoblastic and endothelial or mesenchymal. In Fig. 22 hemocytoblastic infiltration in the lower part of the field, endothelial or mesenchymal in the upper part. Magnifications: Fig. 21, × 100; Fig. 22, × 200.
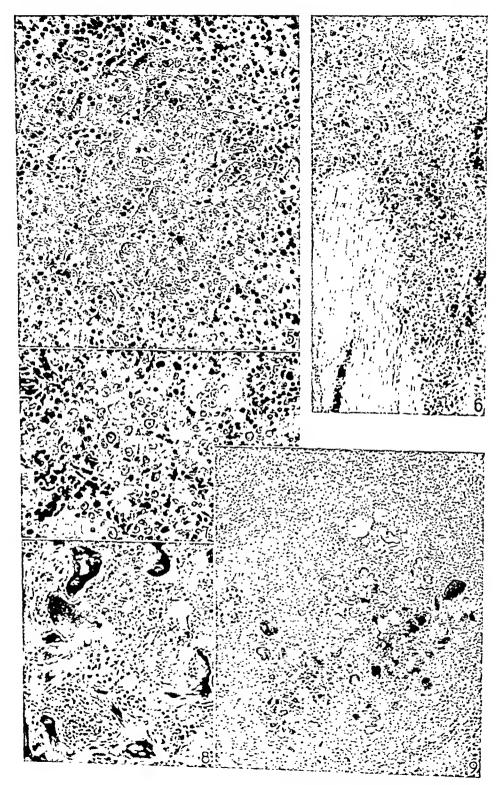
Fig. 23. (No. 3489.) Hypertrophy and hyperplasia of the endothelium of a sinusoidal capillary of the bone marrow. Detached endothelial cells and hemocytoblasts occupy the sinusoid that is surrounded by myelocytes. × 400.

Fig. 24. (No. 3489.) An area of bone marrow with formation of myelocytes from endothelial or mesenchymal cells. × 350.
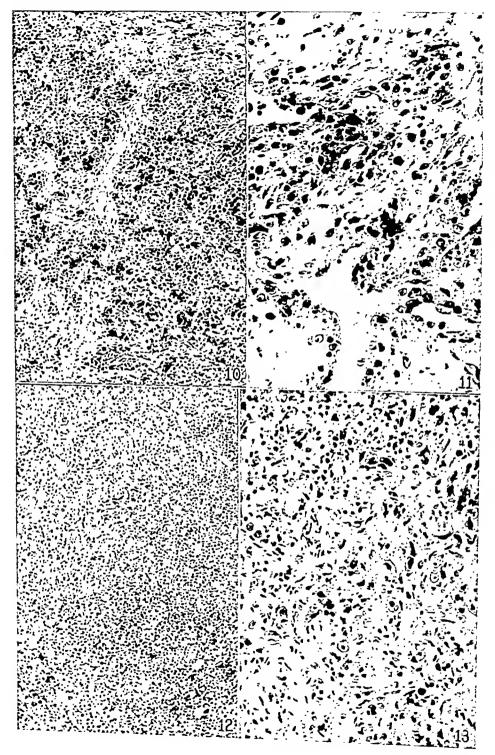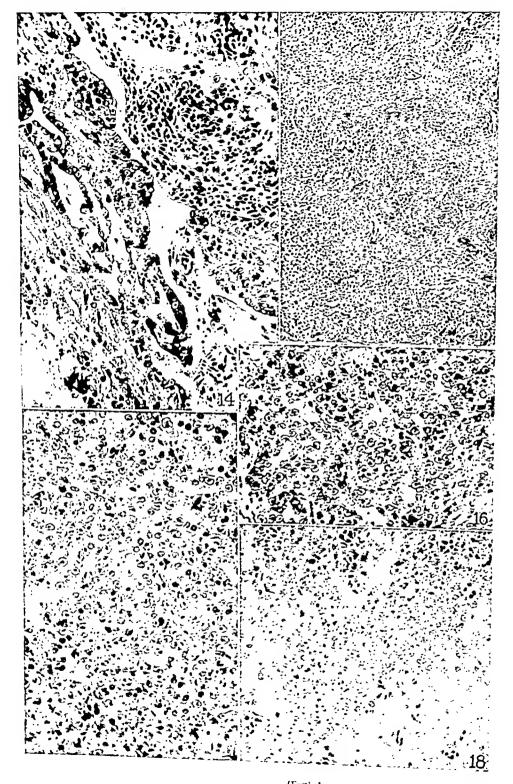
PLATE 35
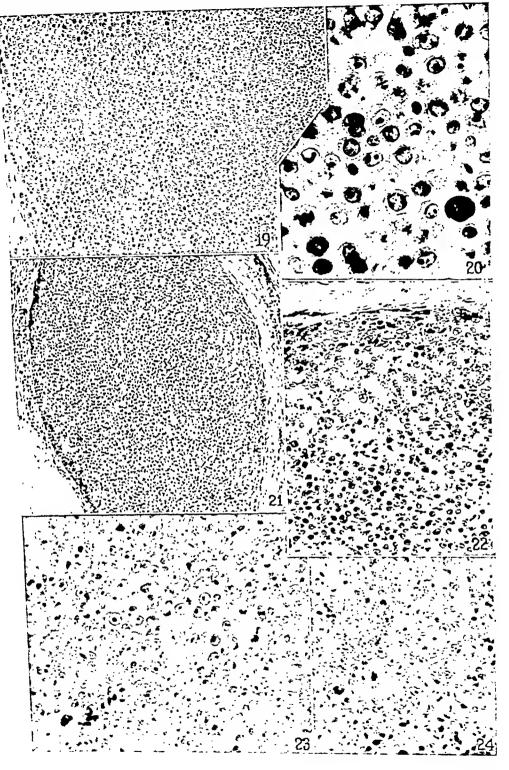
QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION

THE RÔLE OF MULTIPLE REACTIVE GROUPS IN ANTIGEN-ANTIBODY UNION AS ILLUSTRATED BY AN INSTANCE OF CROSS-PRECIPITATION*

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The writers have been studying an antigen-antibody system in which the antigen was the red dye, R-salt-azo-benzidine-azo-crystalline egg albumin[1] (1, 2), fractionated to the extent that it reacted only exceptionally with anti-egg albumin sera. However, as stated previously (2), the original specificity was not entirely abolished, since antisera to the dye protein yielded precipitates with crystalline egg albumin. A quantitative comparison of the dye-antidye and egg albumin-antidye reactions disclosed great differences, as will readily be seen from Table I and the corresponding Fig. 1. In the latter the solid curve (I) represents the amount of antibody ([total N − antigen N] × 6.25) precipitated per cubic centimeter from an antidye rabbit serum by varying amounts of dye protein; the broken curve (II) shows the amount of antibody precipitated by varying amounts of egg albumin from the same serum, while the dotted curve (III) represents antibody precipitated by egg albumin from anti-egg albumin of similar precipitin content. The conclusions regarding antigen-antibody interaction drawn from the data will be discussed below.

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* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

[1] Referred to throughout this communication as "dye protein" or "dye."

EXPERIMENTAL

When small quantities of antigen are added to antibody in the dye-antidye[2] system, or egg albumin (E.A.)-anti-E.A.[3] system, antigen cannot be detected in appreciable amounts in the supernatant from the specific precipitate until the equivalence point is reached; that is, the point at·which the sum of the molar concentrations of both components is at a minimum. However, in the E.A.-antidye system small amounts of E.A. often fail to yield precipitates with antidye (although larger amounts do) but may be demonstrated by the addition of anti-

TABLE I

Analytical Data on Direct and Cross-Precipitin Reactions

Antigen added per cc. antiserum	Protein precipitated per cc.		Reaction of supernatant with anti-E.A.	Antibody precipitated per cc.		Reaction of supernatant with antidye
	Anti-E.A. Solution B. 157	Serum 7.42 antidye		Anti-E.A. Solution B. 157	Serum 7.42 antidye	
mg.	*mg.*	*mg.*		*mg.*	*mg.*	
E.A.						
0.007	0.10		—	0.09		
0.017	0.21		—	0.19		
0.033	0.44		—	0.41		
0.067	0.67		±	0.60		
0.075		0.07	++		0.06	
0.10	0.75		+±	0.68		
0.15		0.10	+++		0.09 (A)	
0.167	0.42		+++	0.36		
0.72		0.61	++++		0.55	
1.50		0.69			0.63	
Dye protein						
0.075		0.75			0.68	—
0.15		0.78			0.70 (B)	+
1.0		0.19			0.16	

E.A., which quickly forms a precipitate (see also Table I, Columns 4 and 7). This affords evidence for the dissociation or greater solubility of the E.A.-antidye complex and for the absence in the antidye serum of the characteristic antibody

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[2] Antidye is defined in this article as the antibody produced in rabbits on injection of preparations of R-salt-azo-benzidine-azo-crystalline egg albumin which have been fractionated so as to remove substantially all material precipitating in anti-egg albumin sera.

[3] Anti-egg albumin is defined as the antibody produced in rabbits on injection of crystalline egg albumin.

to E.A. That solubility alone does not account for the observations summarized in the table and the figure is indicated by the continued increase in precipitation at concentrations of E.A. far higher than those necessary for combination with all of the antibody, just as addition of an ion in common drives back the dissociation of a sparingly soluble salt and increases precipitation.

The quantitative determinations of precipitin were made by the method previously described (2). In the case of the E.A.-anti-E.A. system (in which the antibody solution contained the total globulin from three different anti-E.A. rabbit sera) the total amount of E.A. used was deducted from the precipitable protein as far as the equivalence point, beyond which E.A. first began to appear

Mg. Antibody precipitated

Mg. Antigen added
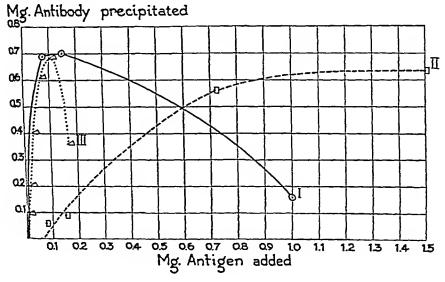
FIG. 1

in the supernatant. In the single determination in Table I and Fig. 1 in the region of the inhibition zone, the excess of E.A. in the supernatant was determined by setting up an aliquot with an excess of the same antibody solution and calculating the amount of E.A. from the total protein precipitated, as previously described in the case of a specific polysaccharide (3). From this value the amount in the original precipitate was obtained. In the case of the E.A.-anti-dye system the amount of antibody precipitated was calculated by deducting from the total protein precipitated one-twelfth of its value, it having been found in this laboratory that the equivalence point ratio of E.A. to anti-E.A. is 1 to 11 (cf., however, Culbertson (4)), so that 12 parts of precipitate would contain 1 of antigen. In the E.A.-antidye system this method of calculation involves the

assumption that the composition of the precipitate is constant over the entire range. This would appear justifiable as a first approximation, since the E.A.-antidye cross-reacting system differs from the two homologous systems in showing no inhibition zone with excess antigen. There is thus at least one less compound possible in the cross-reaction, the writers having shown, in agreement with Arrhenius' belief, that a soluble compound is formed in the inhibition zone of homologous reactions (5, 1).

The supernatant from the precipitate marked (A) in Table I was divided into 2 aliquot portions and precipitated with a slight excess of dye. 0.60 mg. of antibody was recovered, calculated per cc. of the original serum. This, added to the 0.09 mg. precipitated by the E.A., equals 0.69, in excellent agreement with (B), the total found with the dye alone.

Similar results were obtained with other sera, although the form of the E.A.-antidye curve was somewhat different in each case. Serum 8.01, for example, contained 1.94 mg. of antidye per cc., but of this only 1.09 mg. was precipitable by E.A. With 25.6 mg. of E.A. 1 cc. of this serum precipitated 0.97 mg. of antibody, while the supernatant, with 0.05 mg. of the dye, yielded 0.85 mg. of antibody, calculated to the original volume, or a total of 1.82 mg. as compared with 1.94 mg. found directly. In this serum, then, even a large excess of E.A. failed to inhibit the dye-antidye reaction markedly, although greater inhibition was shown in other sera. In this serum, also, equilibrium in the E.A.-antidye system was established more slowly than in Serum 7.42, so that in subsequent work the E.A.-antidye mixtures were allowed to stand for 2 days before completing the analysis. The fact that the amount of antidye precipitated by both antigens in succession corresponds so closely to the amount of antidye found with the dye alone indicates that appreciable dissociation of the E.A.-antidye did not occur during the relatively short time consumed by the washings at 0°C. Supernatants from the maximum dye-antidye precipitate often yielded, when tested with E.A., an amount of additional precipitate equivalent to 0.05 to 0.11 mg. of anti-E.A. per cc. While these quantities fall within the limits of error of the method used, the occurrence of these precipitates is not inconsistent with the interpretation discussed below.

In Table II are given the data resulting from the addition of E.A. in successive portions to the antidye Serum 1.14 and a mixture of Sera 1.15 and 1.46. It was shown (2)[4] that Serum 1.14 contained 3.33 mg. of specifically precipitable antibody per cc. at the time of bleeding, and 3.06 mg. 6 months later. When used in the present experiment, 1½ years after the bleeding, the antidye content was unchanged; namely, 3.19 mg. per cc. The mixture of the other two sera, which originally contained 1.53 and 1.56 mg. of precipitable antibody, yielded 1.69 mg. The sera contained 0.01 per cent of merthiolate. To 5.0 cc. of Serum 1.14 and 10.0 cc. of the mixture of Sera 1.15 and 1.46 were added 0.20 cc. of saline and

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[4] Heidelberger, Kendall, and Soo Hoo (2), p. 150.

0.20 cc. of a 1:1000 solution of crystalline egg albumin, with thorough mixing. In the first three or four runs the tubes were allowed to stand for 24 hours at room temperature and then 24 hours at 0°, but in the later runs a period of 48 hours at 0° was allowed in order to minimize the risk of bacterial growth in spite of the antiseptic present. The tubes were whirled in the refrigerating centrifuge[5] and the precipitates were analyzed as described in a previous article (2), using 4 and 3 cc. of chilled saline for the first and second washings, since the runs were carried out in 15 cc. centrifuge tubes. 5.0 and 10.0 cc. of the respective supernatants were then treated as before, with a final increase in the amount of E.A.

TABLE II

Serial Additions of Egg Albumin to Antidye

Amount of E.A. nitrogen added	Total antidye N: 2.57 mg.			Total antidye N: 2.70 mg.		
	Serum 1.14			Mixed Sera 1.15 and 1.46		
	Total N precipitated	Antibody N precipitated (calculated)	E.A. N precipitated (calculated)	Total N precipitated	Antibody N precipitated (calculated)	E.A. N precipitated (calculated)
mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.031	0.314	0.288	0.026	0.380	0.348	0.032*
0.031	0.280	0.257	0.023	0.294	0.269	0.025
0.031	0.210	0.192	0.018	0.256	0.235	0.021
0.062	0.226	0.207	0.019	0.264	0.242	0.022
0.155	0.134	0.123	0.011	0.138	0.126	0.012
0.62	0.258	0.236	0.022	0.308	0.282	0.026
2.98	0.144	0.132	0.012	0.164	0.150	0.014
2.98	0.144	0.132	0.012	0.148	0.136	0.012
2.98	0.054	0.049	0.005	0.064	0.059	0.005
0.015 dye N	0.076	0.068		0.132	0.117	

* Actually a trace of E.A. was found in the supernatant with anti-E.A., indicating that this figure should not have been >0.030. These sera, or one of them, possibly contained small amounts of anti-E.A.

added. Even the first supernatants reacted with anti-E.A., again showing the E.A.-antidye precipitate to be dissociated. The nitrogen values found by the micro Kjeldahl method were calculated as in Table I, assuming a constant 1:11 antigen-antibody ratio for the precipitate. When only traces of antibody were finally precipitated by the E.A. added, final runs were made with small amounts of dye. In both cases the total amount of antidye precipitated was within 10 per cent of the total indicated by the original determination with the dye itself, which may be taken as a satisfactory agreement considering the large numbers of dilution factors and analyses involved.

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[5] Manufactured by the International Equipment Co., Boston.
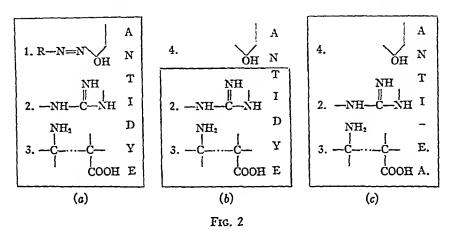
DISCUSSION

Although the antigen used in these experiments, R-salt-azo-benzidine-azo-crystalline egg albumin, was fractionated until it was practically non-reactive with antisera to crystalline egg albumin over a wide range of concentration, antisera produced with the dye can be largely deprived of their antibody content by suitable additions of crystalline egg albumin. There is no *a priori* reason why the antigen could not be split in the animal body so as to form the same antibody as does egg albumin itself, but it is believed that the experiments described show conclusively that practically all of the antibody which reacts with the egg albumin is the antibody formed as a result of the specific antigenic stimulus due to the dye protein itself. There is thus no necessity for assuming the presence of antibody other than antidye. This conclusion is based on (1) the totally different quantitative aspect of the egg albumin-anti-egg albumin and egg albumin-antidye reactions (Fig. 1); (2) the similarity of the egg albumin-anti-egg albumin and dye-antidye reactions (Fig. 1); (3) the additivity of successive egg albumin-antidye and dye-antidye precipitations; and (4) the fact that at all concentrations of egg albumin in the egg albumin-antidye cross-reaction free egg albumin may be detected in the supernatant by addition of anti-egg albumin.[3] This result would be expected if the combination between egg albumin and antidye were a loose, highly dissociated one. Consistent with this interpretation are the large amounts of egg albumin required to force the egg albumin-antidye complex out of solution—amounts far in excess of those required to throw an homologous reaction completely into the inhibition zone, whether one is dealing with the egg albumin-anti-egg albumin or the dye-antidye system. Moreover, in the two homologous reactions antigen cannot be detected in the supernatant until after the equivalence point has been reached, showing that in these cases the antigen-antibody complex is almost undissociated.

The occurrence of this one-sided cross-reaction may be explained by the assumption that in the dye protein there still remain minor antigenic groupings common to egg albumin itself. These may be somewhat masked by the dominant azo groupings which determine the homologous specific reaction, thus preventing the *in vitro* union of dye and anti-egg albumin. However, in the animal, these minor

antigenic groups might become reactive and occasion, in the antidye molecule, the formation of correspondingly minor reactive groups, through which antidye could react with egg albumin as well as with the dye itself.

The following diagrams may make this interpretation clearer:

FIG. 2

Grouping 1, in accordance with Landsteiner's findings, is the dominant antigenic group in the dye protein, while 2 and 3 are other arbitrarily chosen groups of which the steric configuration is characteristic for egg albumin. The dominant reactive group on the antidye molecule would be one evoked in the animal by virtue of the dominant antigenic group (cf. Breinl and Haurowitz (6), Mudd (7)). The resulting dye-antidye combination might therefore be represented by Fig. 2 a, and would be expected to be relatively firm and undissociated. Other less important groups on the antibody molecule might be expected to result from Groupings 2 and 3, characteristic of the egg albumin portion of the dye molecule. Thus crystalline egg albumin, with its dominant antigenic Group 4, arbitrarily chosen by analogy with Group 1, might be expected to react with antidye by virtue of Groupings 2 and 3. The union could reasonably be expected to be a relatively weak, highly dissociated one, since there would be no anti-4 in the antidye, and the egg albumin contains no Grouping 1 to react by virtue of the anti-1 present. This is represented in Fig. 2 b. Again, crystalline egg albumin, with its dominant

antigenic Group 4, would be expected to form the observed firm, undissociated union with anti-egg albumin, containing anti-4, as represented in Fig. 2 *c*.

These views derive from those expressed by Landsteiner and van der Scheer (8) and are somewhat analogous to those of Bergmann on the combination of an enzyme with its substrate (9).

In accordance with this interpretation egg albumin would react with the antibody to the dye protein by virtue of the minor antigenic groupings common to egg albumin and dye protein. The antibody would, however, be "antidye" as a result of the stimulus exerted on the animal by the dominant antigenic dye grouping, and it is shown in this communication that "antidye" has totally different reactivities from those of "anti-egg albumin." For example, egg albumin and anti-egg albumin yield a firm, undissociated combination (Fig. 1, Curve III; Table I). With small additions of egg albumin to anti-egg albumin it is impossible to detect antigen in the presence of excess antibody, and it is only on increasing the amount of egg albumin beyond that required to reach the equivalence point that antigen appears in the supernatant. The interaction of dye protein and antidye is of the same type (Fig. 1, Curve I; Table I). While it is possible that antisera to the dye contain minimal amounts of true anti-egg albumin resulting from undetected traces of egg albumin in the antigen, the reaction between egg albumin and antidye is qualitatively and quantitatively so different from the homologous reactions that it must be concluded that the reactive antibody is the antidye itself. The differences between the homologous and cross-reactions would seem therefore be due to the firm union brought about by the dominant antigenic and antibody groups in the first instance and to the loose, highly dissociated union resulting in the latter case from the reaction of minor groupings alone.

In the foregoing discussion "antidye" has been considered as a single entity since the experimental data can be most simply treated and presented on this basis. One may, at the other extreme, conceive of a series of "antidyes," all possessing the grouping anti-1 but differing, some by the absence of groupings anti-2 and anti-3, some in their relative proportion. This would perhaps account for the differences in the dissociation constants calculated for different

sera, but it might also be true for the antidye molecules in a single serum. However this may be, it does not affect the conclusion that the reacting antibody in the egg albumin-antidye system is antidye, and that the complex is relatively highly dissociated. It should not be difficult, by the quantitative method, to test the validity of the underlying principle in other cases of cross-precipitation. The partial precipitation of Pneumococcus III antipolysaccharide by partial hydrolysis products of the specific carbohydrate is being studied, as well as an instance of cross-precipitation due to species interrelationships.

A close analogy suggests itself between the instance studied and that observed by Landsteiner and van der Scheer with azo proteins derived from o-aminobenzenesulfonic acid and o-aminobenzoic acid (10), and also to the relation between Type II pneumococcus and Type V (Subgroup II a) (11). The egg albumin-antidye system, however, is evidently simpler than that studied by Avery, Goebel and Babers with α- and β-glucosido-azo proteins (12), or the cross-reactions between the Type B Friedländer bacillus and Type II pneumococcus (13).

The writers believe that their data can be qualitatively explained most simply on the basis of the laws of classical chemistry, assuming antidye as the sole reactive antibody, with a low dissociation constant for the homologous reaction and a high dissociation constant for the egg albumin-antidye reaction. The quantitative formulation of the cross-reaction data in terms of these laws leads to definite values of the dissociation constants which vary from serum to serum, but the calculations involve the making of assumptions which the writers prefer to test more fully.

SUMMARY

1. Antisera to R-salt-azo-benzidine-azo-crystalline egg albumin give precipitates with crystalline egg albumin by virtue of their anti-dye content.

2. The quantitative course of the reactions with increasing amounts of antigen is very similar for the dye-antidye and egg albumin-anti-egg albumin systems, but differs markedly for the cross reaction between egg albumin and antidye.

3. A possible explanation for the occurrence of this one-sided cross-reaction is given in terms of reactive groupings on the antigen and antibody.

4. A qualitative expression of the course of the cross-reaction is given in terms of the laws of classical chemistry.

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THE HISTOLOGY OF EQUINE ENCEPHALOMYELITIS

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The severe epidemic of equine encephalomyelitis raging along the
Atlantic seaboard in August and September, 1933, afforded an oppor-
tunity of studying the histology of the naturally occurring malady,
the causative virus of which appeared to be serologically distinct
from that responsible for the rather similar disease in California
(TenBroeck and Merrill, 1933). At that time an experimental study of
the western type of encephalomyelitis was under way, the histological
features having been only cursorily dealt with by Meyer and his
colleagues (1932). The opportunity is now taken of comparing the
lesions of the two infections.

Histological Findings in Horses

(a) Equine Encephalomyelitis in New Jersey and Virginia

The organs of eight field cases were examined histologically; all the
animals were killed by bleeding or anesthesia, and the tissues deposited
in fixatives within half an hour or so of death. The duration of
nervous symptoms varied from a few hours to several days. Macro-
scopically the nervous system was congested and often somewhat
edematous. The microscopical lesions, of identical nature in all
eight cases, were of the same general type as those in monkey polio-
myelitis, though their distribution was vastly different. In view of
their character it is perhaps necessary to emphasize the statement
that on culture the majority of the brains were sterile.

Most prominent in the nervous system with the low power of the microscope
was the cellular infiltration (Fig. 1). In many regions polymorphonuclear leuco-
cytes, distributed diffusely and in small or large foci, swarmed in the tissues (Fig.

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2); with these was associated very early microglial proliferation giving rise to many rod cells. In rare foci composed almost wholly of polymorphonuclear leucocytes the tissues appeared on the verge of actual softening. In other areas microglial elements predominated, though nearly always leucocytes were present and were often numerous. In either case perivascular infiltration might be inconspicuous; commonly, however, cuffs one to many cells deep surrounded the local vessels (Fig. 1). They consisted almost wholly of neutrophil leucocytes, many karyorrhectic, or of small and large lymphocytes associated with a variable number of neutrophils and rare eosinophils. Polymorphonuclear leucocytes often overflowed into the adjacent nervous tissues, while among the lymphocytes mitoses were numerous. The meninges overlying areas of intense change were often infiltrated to a marked degree with similar cells; on the other hand, over many areas similarly affected, and over those showing slighter changes, meningeal infiltration was wholly wanting. Only occasionally was infiltration noted in the choroid plexuses.

When resort was not had to bleeding, vascular congestion was pronounced and small perivascular hemorrhages not infrequent. In the congested state of the vessels the latter observation probably has little significance, as hemorrhage might well be produced by trauma during removal of the brain. Signs of reaction indicating extravasations of some standing were never seen.

The essential lesion, and one occurring in a few places in the absence (as yet) of any marked cellular reaction, was acute necrosis of nerve cells, which appeared as shrunken eosinophilic structures devoid of nucleus or showing pyknotic nuclear remains (Fig. 2). In severely affected cortical areas cellular architecture was partly disorganized. Often the necrotic cells were in process of lysis by neutrophils plastered on their surfaces (Figs. 3 and 4). In other instances, more frequently perhaps in brain stem and cord, neuronophagia by microglial cells was observed. Less severe degenerative changes in the neurons included vacuolation of the cytoplasm and invasion of the vacuoles by neutrophils, occasionally in the presence of almost normal Nissl substance. Simple chromatolysis and nuclear eccentricity commonly affected cells of the cranial nerve nuclei and anterior horns of the spinal cord. In most of the affected areas, whatever their site, many of the neurons appeared normal. Nuclear inclusions (Fig. 9), much less numerous than in the guinea pig but of the same type and distribution, will be described in connection with the changes in that animal, in which fuller study was made; they bore considerable resemblance to those associated with Borna disease.

Neuroglial nuclei were often greatly swollen and very pale; no inclusions were detected therein or in mesodermal elements.

The foregoing alterations were most pronounced in the cerebral cortex. Although many unaffected areas existed side by side with zones of severe destruction, and in individual cases the anterior frontal or occipital regions or the cornu Ammonis might be relatively lightly involved, no major subdivision was immune from attack or was consistently more affected than another. The lesions mani-

fested no predilection for a particular cortical layer. The cornu Ammonis and rhinencephalic cortex never showed the massive necrosis to be described in the guinea pig, etc. The olfactory bulb was not affected with exceptional severity. In the subcortical white matter foci of neutrophil infiltration or microglial increase occurred much more rarely than in the grey matter, though more frequently than in most virus diseases. Here, sometimes, massed polymorphonuclear leucocytes collected in the nervous tissues around part of the circumference of an artery not itself infiltrated; occasionally the impression of incipient softening was given. The caudate and lenticular nuclei usually showed less severe changes than did the cortex; the optic thalamus and hypothalamic regions were fully as badly damaged. In two instances the fiber bundles entering the putamen were packed with leucocytes, while the grey matter of the nucleus had largely escaped involvement (Fig. 6). In five cases the brain stem and cervical cord were definitely less affected than the higher centers; the intensity of the changes often varied greatly at different levels and attained a maximum in the grey matter around the ventricular system, in the pontile nuclei, and in the olives. At some levels the lesions appeared to be very early and consisted mainly in nerve cell degeneration and stuffing of the capillaries with polymorphonuclears, which could be seen passing through their walls into surrounding structures. In the cord both anterior and posterior horns were involved, but nerve cell destruction took place chiefly in the former. Only mild changes were present in the cerebellar cortex, and in two animals no lesions were detected here; the cerebellar nuclei usually suffered more severely. In all the horses the Gasserian ganglia were normal.

No significant abnormality was detected in the lungs, liver, spleen, kidneys, adrenals, salivary glands or lymph nodes. In one advanced case the centers of the liver lobules appeared to be in the preliminary stages of acute necrosis; the liver cells were shrunken and eosinophilic with pyknotic nuclei, the Kupffer cells greatly enlarged and increased in number, and numerous neutrophils occupied the sinuses and central veins. At the margins of these areas some fatty infiltration was apparent and the liver cell nuclei were often greatly enlarged. No virus was present in the organ, nor were bacteria demonstrable in sections. It does not appear probable that the changes were the direct result of virus activity. In several instances the cervical lymph nodes were greatly congested but showed no other abnormality.

To summarize, the eastern type of equine encephalomyelitis is characterized histologically by an unusually diffuse and intense acute inflammation affecting most territories of the central nervous system but more particularly the grey masses. Nerve and neuroglial cell degeneration is undoubtedly primary, though accompanied or speedily followed by heavy tissue infiltration with polymorphonuclear leucocytes and reactive changes in the microglia. In the nerve cells

nuclear inclusions occur rather similar to those in Borna disease. Perivascular infiltration may be predominantly polymorphonuclear or wholly mononuclear. Meningeal infiltration is clearly secondary to changes in the underlying nervous substance. No significant changes are consistently present in organs other than the nervous system.

The foregoing picture was accurately reproduced in a horse inoculated intracutaneously (in the dorsal region) with the brain of a field case.

(b) Lesions in a Horse Infected with the Western Virus

This horse, inoculated intranasally and intracutaneously (in the area of supply of the fifth cranial nerve) by Dr. Erich Traub, was killed on the 12th day, after having exhibited nervous symptoms for 3 days. It presented lesions which individually could not be distinguished from those described above. They were, however, far less numerous and intense. But few areas of the cerebral cortex were involved; the chief sites of injury were the olfactory bulbs, the optic thalamus and hypothalamic region, and the brain stem. Only a few nerve cells were acutely necrotic and undergoing neuronophagia; in the areas named, polymorphonuclear tissue infiltration, microglial proliferation and mononuclear infiltration were less intense than in the eastern cases. In this horse the Gasserian ganglion showed mononuclear interstitial infiltration.

Histological Findings in the Calf, Sheep and Dog

These animals were inoculated intracerebrally with the eastern virus by Dr. C. TenBroeck. The calf developed pronounced symptoms and was killed 72 hours after inoculation. The sheep died 117, and the dog 84 hours after injection. In all, the microscopical picture was essentially that depicted above; the minor differences observed necessitate only brief mention.

In the calf, changes in the cerebral cortex were in places even more intense than in horses, with acute necrosis of a larger proportion of neurons. In parts of the cortex, basal ganglia, brain stem and anterior horns of the spinal cord small foci of softening crammed with polymorphonuclear leucocytes might be discerned. In most regions, however, lesions were less severe. Perivascular cuffing was

everywhere mainly or exclusively mononuclear.   The cerebellum was unaffected. Meningeal infiltration, though marked over some regions of the cerebral hemispheres, may have been due in part to the introduction of foreign nervous tissue, since over severely affected parts of the brain stem and cord it was often wholly wanting.

Lesions in the sheep were rather less severe than in the calf and, probably as a result of greater duration of the inflammatory process, the infiltrating polymorphonuclear leucocytes were highly karyorrhectic. Necrotic nerve cells were frequent in parts of the cortex and in the anterior horns of the cord.   Perivascular cuffs were solely mononuclear. Mild lesions obtained in the cerebellar cortex and more marked lesions in the dentate nucleus, etc. Meningeal infiltration was in most areas inconspicuous.

Changes in the dog resembled those in the sheep except that at only a few levels of the brain stem and cord did marked lesions prevail. The cerebellum was unaffected.

Histological Findings in the Guinea Pig

The guinea pig is the laboratory animal of choice for the study of equine encephalomyelitis. Lesions were substantially the same whether the virus was introduced intracerebrally, intramuscularly, subcutaneously or intradermally, and whether the eastern or the western virus was employed.   Any differences apparent in infections with the two viruses could well be ascribed to greater virulence of the one and the lesser virulence of the other.   It happened that more intense lesions were present in guinea pigs inoculated peripherally than in those inoculated intracerebrally; probably the longer incubation in the former allowed wider dissemination and greater multiplication of the virus.   On the whole the lesions produced by intracerebral inoculation of the western strain were comparable with those following peripheral inoculation of the eastern organism.   The more or less uniform symptomatology was little indication of the degree of microscopic abnormality, which increased with longer duration of the developed disease.   At the time when nervous symptoms were first manifest only early degeneration of those nerve cells later most severely affected was present: vascular and interstitial inflammation were absent or at a minimum.   It was at this stage that many of the animals inoculated intracerebrally with the eastern virus died.

In the fully developed disease, lesions in the cerebrum were of fairly uniform distribution, with a decided tendency to greater intensity in the cornu Ammonis

and rhinencephalic cortex. In less severe and acute cases (histologically), perivascular infiltration with small and large lymphocytes affected a variable number of vessels; in their vicinity and elsewhere in the nervous substance microglial proliferation obtained (Fig. 7). In more acute cases polymorphonuclear leucocytes, many karyorrhectic, appeared in the perivascular infiltrate and constituted an overwhelming majority of the tissue infiltrate Some proliferation of vascular adventitial cells occurred. In several cases massive acute necrosis involved large parts of the cornu Ammonis or rhinencephalic cortex, or both regions, and vast numbers of polymorphonuclear leucocytes congregated around the destroyed neurons (Fig. 8). Meningeal infiltration rarely amounted to more than the presence of a few mononuclear cells, with some polymorphonuclears, in the depths of the fissures; occasionally it was marked over an area of cortex showing very intense changes.

Lesions in the brain stem and spinal cord were variable in degree and were not necessarily most evident in animals inoculated peripherally. Acute necrosis often struck cells of the cranial nerve nuclei, olivary bodies and anterior horns; in the cerebellum Purkinje cells suffered with much less frequency. Cellular infiltration as in the cerebrum, and sometimes neuronophagia followed; in the cord the former was limited usually to the grey matter, but occasionally a focus of proliferated microglial cells lay in the white matter. Less severe nerve cell degeneration taking the form of tigrolysis and nuclear eccentricity was common. The Gasserian and spinal ganglia were normal in all cases inoculated intracerebrally; after intramuscular inoculation only some mononuclear infiltration with a few polymorphonuclears was noted in the nerve roots.

Careful search invariably demonstrated nuclear inclusions in nerve cells, neuroglial cells, mesothelial cells of the pia-arachnoid and adventitial cells of the vessel walls, in this order of frequency. In the nerve cells of the brain stem, single or multiple bodies were relatively frequent at an early stage preceding marked tissue and perivascular infiltration (Figs. 10–12); six or seven cells containing inclusions might be seen in a single section. In other neurons they were more seldom found either then or later; in the cornu Ammonis they were not more numerous than in the general cortex, and in both situations definitely less common than in the brain stem. Affected nerve cells usually showed other signs of degeneration, if only incipient, but inclusions were not detectable in dead or moribund elements. The round or slightly oval, strongly oxyphilic bodies of sharp contour bore considerable resemblance to those in Borna disease; they were, however, often rather larger, less often showed a center paler than the periphery or a central vacuole-like structure, and possessed perhaps slightly less sharp contours. Normal weakly oxyphilic nuclear material was often diminished in amount or absent, and the inclusions were differentiated from the feebly oxyphilic masses normally present (nucleonephelium of Saguchi, 1930) by greater regularity and definition of outline and deeper coloration with the acid dye. The sharpness of definition of the bodies was greater in cases with a longer incubation period, and was less with virus strains producing symptoms within a short time of inoculation.

Nuclear inclusions in the other elements mentioned took the form of very tiny, less strongly oxyphilic spherules. In glial cells swelling and pallor of the nucleus and margination of basophilic chromatin often coexisted, especially in areas where marked changes were present. The tiny bodies were difficult to demonstrate, and but for the presence of definite bodies in the nerve cells would have been disregarded. Similar granules did not occur in normal animals and it seemed legitimate to accept them as analogous to the abnormal nuclear products met with in the neurons; they bore roughly the same dimensional relation to the enclosing nuclei as did the larger bodies to the nerve cell nuclei.

The lungs frequently showed pathological changes. Macroscopically one or more lobes appeared solid and plum-colored; or the organs were deeply congested with darker plum-colored areas of serpiginous outline indicated on the pleural surface. Edema and petechial hemorrhages might be present. Histologically, large areas of collapse existed and enclosed bronchopneumonic areas; the latter also occurred independently of atelectasis. In the bronchial exudate of such cases a great variety of bacteria might commonly be demonstrated. A second type of pulmonary change took the form of partial atelectasis with pronounced interstitial change and no exudate in the bronchi or alveoli. The alveolar walls were thickened by a mononuclear infiltrate containing scanty neutrophils, and intense perivascular and peribronchial infiltration with similar cells was evident. Small foci of proliferation and desquamation of the alveolar epithelium occurred. In some cases both types of pulmonary change coexisted.

In two animals necrosis of isolated liver cells with some polymorphonuclear infiltration existed in the apparent absence of any excitant other than the infecting agent employed. In one case moderate interstitial mononuclear infiltration was present in the salivary glands. The sinuses of the spleen usually contained an excessive number of neutrophil leucocytes. In two guinea pigs isolated cells of the adrenal cortex were necrotic.

In the guinea pig, then, the virus of equine encephalomyelitis (eastern or western strains) produces a histological picture fairly comparable with that in horses, except that the maximum injury in the cerebrum is inflicted upon the cornu Ammonis and rhinencephalic cortex. Nuclear inclusions of the type encountered in Borna disease occur chiefly in the brain stem, and, as is not the case in the horse, tiny oxyphilic bodies of apparently similar nature are less frequently observed in glial and mesodermal elements. Unlike the horse, this animal commonly presents pneumonic lesions in the lungs, sometimes those of a secondary bronchopneumonia, sometimes of the type which is now recognized as typical of virus infections. Minor changes less certainly due to virus action are sometimes found in other organs.

Histological Findings in Rabbits and Mice

The virus of equine encephalomyelitis is less pathogenic for the rabbit than for the guinea pig; this is more especially true of the western strains which even on intracerebral inoculation rarely produce more than a temporary illness. The histological picture, though essentially the same as in guinea pigs, is correspondingly less acute. Polymorphonuclear leucocytes are but rarely present in the tissues, where more advanced metamorphosis in the direction of granular corpuscles is permitted in the microglia. Meningeal infiltration is more marked than in the guinea pig and almost wholly mononuclear, as are the perivascular cuffs. Massive acute necrosis is rarely seen in the cornu Ammonis, where, however, long stretches of nerve cells of the pyramidal layer often undergo severe degeneration and exhibit swollen cell bodies completely devoid of Nissl substance; nuclear inclusions here may be particularly large and numerous. Inclusions are seen only in nerve cells.

On intracerebral inoculation the eastern virus produces in mice a very rapidly fatal disease, often killing the animal in 50–60 hours. The scanty lesions are similar in nature and distribution to those of the more rapidly fatal disease in guinea pigs. Massive necrosis may be seen in the cornu Ammonis and rhinencephalic cortex. Inclusions are present only in nerve cells.

Histological Findings in Rabbits Infected with the Virus of Borna Disease

In the brains of three rabbits, lesions similar to those described by Zwick, Seifried and Witte (1927) were much less acute than in infections due to the virus of equine encephalomyelitis.

No polymorphonuclear leucocytes were present in the tissues, though occasionally lymphocytes overflowed from the perivascular cuffs. The latter were composed wholly of mononuclear elements and included a number of plasma cells; the cells of the adventitia sometimes showed active proliferation. Mononuclear meningitis was marked in places, and often accompanied in the subjacent zone by infiltration with round cells and microglial cells and definite enlargement of the subpial glial cells. Active diffuse and focal microglial proliferation in the cortex was associated with marked satellitosis of neurons and sometimes neuronophagia. The rhinencephalic cortex, cornu Ammonis, lateral cortex and region of the third ventricle were particularly affected; in the first, acute necrosis of nerve cells was

noted. In the brain stem small microglial foci and marked perivascular infiltration obtained, together with degenerative changes of moderate severity in some of the nerve cells. Nuclear inclusions were rather smaller than in equine encephalomyelitis, but had a more "solid" appearance and rather sharper contours; they more frequently showed paler central "vacuoles" and indications of a not absolutely homogeneous structure. Only sparse lesions were evident in the cerebellum. The Gasserian ganglia were the seat of marked infiltration, nerve cell degeneration with the formation of nuclear inclusions, and around some neurons multiplication of capsule cells. Typical changes as described by Nicolau, Nicolau and Galloway (1929), and by Zwick, Seifried and Witte (1929) were present in the peripheral nervous system.

COMMENT

The lesions in naturally occurring cases of equine encephalomyelitis are thus typical of acute neurotropic virus diseases, and in nature, though not in distribution, are comparable with those of poliomyelitis in the monkey. Their greater acuity provides a criterion of value in differentiating them from the changes in Borna disease; on the other hand, they are not of the hyperacute type seen in pseudorabies of the rabbit (Hurst, 1933), where death occurs too soon to permit appreciable cellular reaction.

The present study throws little light on possible routes of entry of the virus under natural conditions. Although one horse was killed in the first few hours of nervous manifestations, alterations were already widespread, though at some levels of the brain stem they were at a very early stage. Of the whole series of field cases, not one showed lesions in the olfactory bulbs as marked as did the experimental animal inoculated intranasally and intradermally (in the area of supply of the fifth cranial nerve) with western virus; yet lesions elsewhere in this brain were much less severe than in the eastern cases. It has also been remarked that the distribution of changes seen in field cases in the eastern outbreak was accurately reproduced in a horse inoculated intracutaneously in the dorsal region with eastern virus. These observations, if repeated, might possibly suggest search in the natural disease for a portal of entry other than the nose.

Experimentally, with the eastern strains of virus, the essential histological features of the naturally occurring disease can be reproduced in a number of domestic and laboratory animals. With both eastern and western viruses in the guinea pig and rabbit, and with

the former in the mouse, the chief difference apparent from the histological picture in the higher mammals is the tendency to massive acute necrosis in certain olfactory centers; *viz.*, in the rhinencephalic cortex ventral to the fissura rhinica and in the cornu Ammonis, where, irrespective of whether the virus be introduced intracerebrally or peripherally, whole stretches of pyramidal cells may be destroyed *en masse*. It does not appear to be generally recognized that, wherever else necrosis of neurons may occur, massive necrosis in these situations is of extremely frequent occurrence in the brains of these lower mammals infected with any one of a whole range of neurotropic viruses. In the corresponding diseases of higher animals, although foci of nerve cell destruction may be observed here, massive necrosis is not present. In the case of rabies in the rabbit, Lentz (1909) described the resulting eosinophilic structures containing pyknotic nuclear remains as *"passagewutkörperchen,"* though they are also found in rabbits infected with street virus (Hurst, 1932); I have not seen them in rabies in the cow, dog or monkey. Similar appearances obtain in mice with louping ill (Hurst, 1931 *a*), but not nearly to the same extent in the monkey. I have since seen massive necrosis in the cornu Ammonis of the rabbit with herpetic encephalitis, and in the mouse with yellow fever, while at a recent meeting of the Society of American Bacteriologists (Philadelphia, December, 1933) Dr. L. T. Webster exhibited a lantern slide showing this lesion in a mouse infected with the virus of the St. Louis outbreak of encephalitis. Rivers and Stewart (1928) described similar appearances in Virus III infection in the rabbit. Evidently the lesion is one characteristic of a certain type of animal host, rather than of the action of a particular virus. Now in all these small mammals the olfactory brain is relatively better developed than in higher forms, and is presumably relatively more abundantly supplied with fiber connections. If, therefore, the neurotropic viruses spread by the axis cylinders, for which there is evidence in poliomyelitis, rabies and herpes, we might proffer an explanation of these observations in terms of the probable amount of virus reaching a given locality.

The inclusion bodies present in the nerve cells in equine encephalomyelitis bear considerable resemblance to those described in Borna disease (Joest and Degen, 1909, and others) and in poliomyelitis

(Covell, 1929; Hurst, 1931 *b*).   In view of the recent criticisms of Wolf and Orton (1932) regarding this type of inclusion, particular attention was devoted to the nuclear structure of normal nerve cells.   As various observers, including myself, have pointed out, bodies somewhat resembling these inclusions are often present in the normal nerve cell nucleus.   (Incidentally fixatives containing osmic acid render closer the resemblance; in the present investigation, however, Zenker fixation was employed.)   Wolf and Orton reproduce a number of photographs of these normally occurring bodies as proof that structures indistinguishable from the inclusions of poliomyelitis occur in other pathological conditions in man; since the appearances depicted by them can be seen equally well in normal nerve cells, it is not necessary to resort to pathological human material, necessarily inferior to fresh tissue, to determine the point at issue, whether these normal structures can or cannot be differentiated from the alleged inclusions.

Four photographs showing the inclusions of equine encephalomyelitis (Figs. 9–12) are offered for comparison with those of four normal nerve cells (Figs. 13–16).   In all except Fig. 16 the magnification is the same.   The photographer was instructed to obtain the sharpest picture possible of the intranuclear masses, but was uninformed of the question in dispute.   The pictures in monochrome do not, unfortunately, adequately suggest the deeper and brighter color, in preparations fixed and stained strictly comparably, of the bodies in the cases of encephalomyelitis, but there is obviously a difference between the two groups.   Comparison with Wolf and Orton's photographs shows that the structures in Figs. 13–16 possibly more nearly resemble the inclusions in Figs. 9–12 than do those chosen by the authors named to illustrate their inability to draw a distinction.

The bodies present in normal cells are usually smaller and less acidophilic.   They have less sharp and distinct contours, often appearing rather fluffy in outline, and having a less solid appearance.   They frequently show little projections uniting them to the general, weakly acidophilic, nuclear reticulum.   They never show evidence of internal heterogeneity, as do frequently the bodies described as nuclear inclusions.   Moreover in addition to the greater individuality of the bodies in poliomyelitis, equine encephalomyelitis, etc., the nuclei containing them exhibit a definite tendency to disappearance of

acidophilic material other than that in the inclusions, and a margination of the scanty basichromatin on the nuclear membrane; it is difficult to show all this in a photograph focussing only one plane, yet the general nuclear reticulum in Figs. 9–12 is obviously less in amount than that in Figs. 13–16. This clearing of the nucleus is well shown in the plate previously published to illustrate the similar bodies in poliomyelitis. The appearance of these inclusion bodies represents, therefore, a departure from the normal nuclear structure and is characteristic of certain virus diseases. It is not suggested that the inclusions are necessarily wholly new-formed structures; indeed, in the more acute cases of equine encephalomyelitis in the guinea pig, the appearances suggest that they may be derived from the normal nodal masses which become enlarged, more acidophilic, and freed from the disappearing oxyphilic reticulum. Nor are the bodies considered of greater significance than as indicating in these virus diseases abnormal physicochemical conditions in the nucleus, analogous in a way to those evidenced by solution under various circumstances of the tigroid substance. But it is maintained that these bodies can be distinguished from those occurring in the normal condition and in pathological conditions not due to virus action, when as far as the oxyphilic nuclear components are concerned the normal state is maintained.

SUMMARY

The virus of equine encephalomyelitis (eastern strain) evokes in the horse, calf, sheep and dog an unusually intense encephalomyelitis characterized by acute primary degeneration of nerve cells, the appearance in neurons of the brain stem and elsewhere of nuclear inclusions resembling those in Borna disease and poliomyelitis, polymorphonuclear infiltration in the nervous tissues with early microglial proliferation, and perivascular cuffing with mononuclears and polymorphonuclears in varying proportions. The grey matter is affected more than the white. Lesions may be less marked in the striatum, brain stem and cord than in the cerebral cortex, thalamus and hypothalamic region, and are always of low grade in the cerebellum. Meningeal infiltration is secondary.

Similar changes produced in the horse by the western strain of virus are less intense and extensive.

In the guinea pig, rabbit and mouse, the eastern virus causes an acute encephalomyelitis which, as is usual in neurotropic virus diseases of these lowly species, has a special tendency to affect the higher olfactory centers. In addition to inclusions in the nerve cells, tiny oxyphilic bodies occur with less frequency in the glial and mesodermal nuclei of the guinea pig. In this animal, too, interstitial or broncho-pneumonia may complicate the picture.

In the guinea pig the disease resulting from infection with the western virus may be indistinguishable from that due to the eastern.

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EXPLANATION OF PLATES

PLATE 36

FIG. 1. Eastern type of encephalomyelitis in the horse. Enormously increased cellularity of the tissues due largely to focal and diffuse polymorphonuclear infiltration. An artery shows pronounced perivascular cuffing. Iron alum hematoxylin and Van Gieson. × 71.

FIG. 2. Eastern type of encephalomyelitis in the horse. Comparatively mild polymorphonuclear tissue infiltration in the cerebral cortex. Necrosis of a nerve cell indicated by the arrow. Iron alum hematoxylin and eosin. × 337.

FIG. 3. Eastern type of encephalomyelitis in the horse. Acute necrosis of a nerve cell of the pons. The cytoplasm is markedly eosinophilic, the nucleus absent (in serial section). A polymorphonuclear leucocyte occupies a vacuole in the cell

body, and a second is applied to the surface of the cell. Microglial satellites are also in evidence. Iron alum hematoxylin and eosin. × 1259.

FIG. 4. Eastern type of encephalomyelitis in the horse. Approaching necrosis of a nerve cell of the brain stem. Polymorphonuclear leucocytes have invaded a large vacuole in the cytoplasm; the cell is surrounded by polymorphonuclear and microglial elements. Iron alum hematoxylin and eosin. · ×·718.

PLATE 37

FIG. 5. Eastern type of encephalomyelitis in the horse. This artery in the lenticular nucleus shows little infiltration in the perivascular space but many polymorphonuclear leucocytes are collected in the nervous tissues immediately adjacent. Iron alum hematoxylin and eosin. × 385.

FIG. 6. Eastern type of encephalomyelitis in the horse. Polymorphonuclear infiltration of a fiber bundle entering the putamen; the surrounding grey matter is almost free from infiltration. Iron alum hematoxylin and eosin. × 310.

FIG. 7. Equine encephalomyelitis (western strain) in the guinea pig. Mononuclear perivascular sheathing and microglial proliferation in the cerebral cortex. Intracerebral inoculation. Iron alum hematoxylin and eosin. × 292.

FIG. 8. Equine encephalomyelitis (western strain) in the guinea pig. Massive necrosis in the cornu Ammonis with intense polymorphonuclear infiltration in a bacteriologically sterile brain following intramuscular inoculation. Iron alum hematoxylin and eosin. × 244.
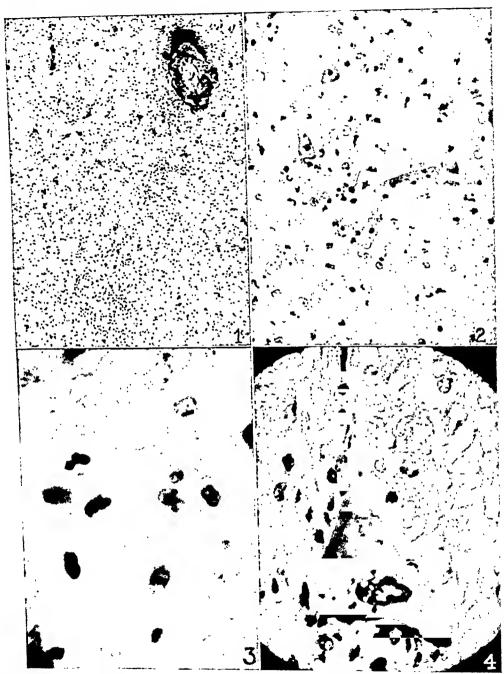
PLATE 38

FIG. 9. Eastern type of encephalomyelitis in the horse. Intranuclear inclusion in a nerve cell of the brain stem. Near the cell is an infiltrating polymorphonuclear leucocyte. Phloxin-methylene blue. × 1888.

FIGS. 10–12. Equine encephalomyelitis in the guinea pig. Nuclear inclusions in neurons of the brain stem. Fig. 10, infection with eastern virus; Figs. 11 and 12, with western virus. Phloxin-methylene blue. × 1888.

PLATE 39

FIGS. 13–15. Nerve cells in brain stem of healthy guinea pigs to show acidophilic nuclear bodies normally present. Phloxin-methylene blue. × 1888.
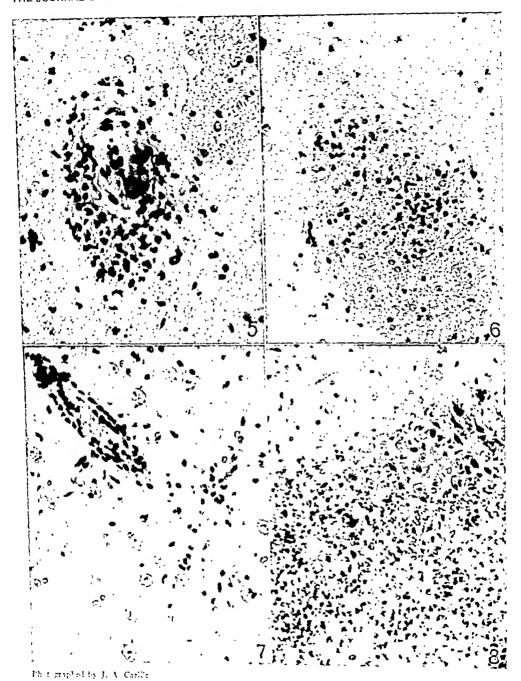
FIG. 16. Normal nerve cell from spinal ganglion of pig. Phloxin-methylene blue. × 851.

(Hurst: Histology of equine encephalomyelitis)

PLATE 37
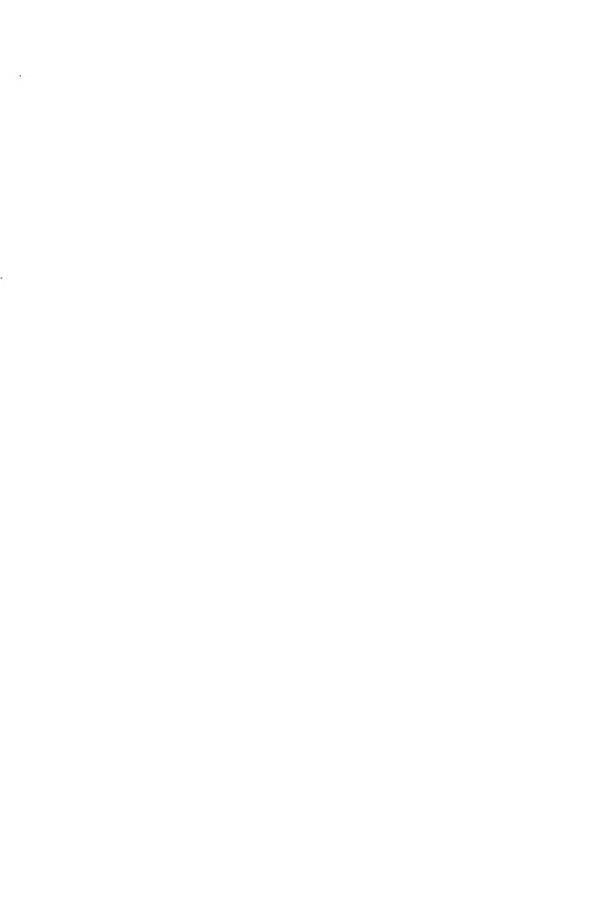
Photographed by J. A. Carlile
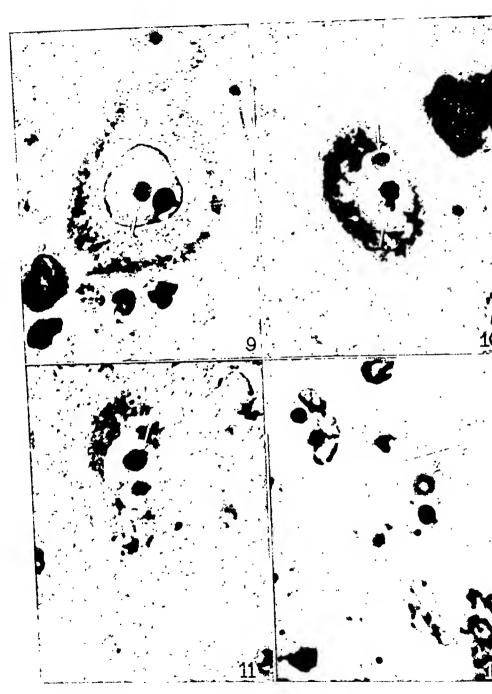
(Hurst. Histology of equine encephalomyelitis)

PLATE 38

(Hurst: History of equine encephalomyelitis)

(Hurst: History of equine encephalomyelitis)
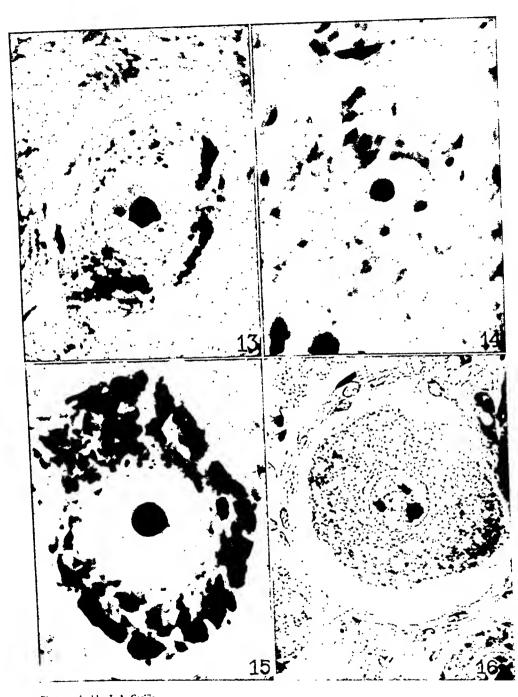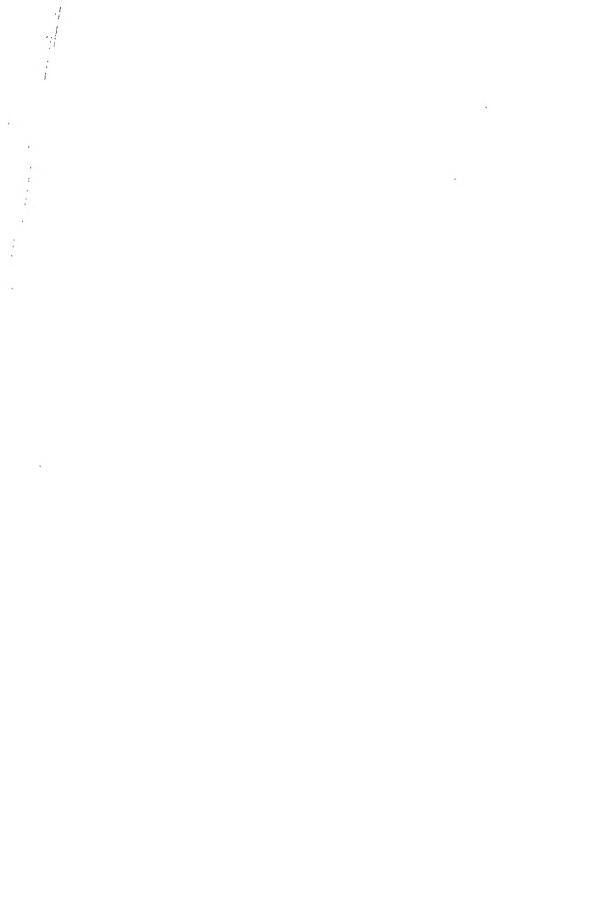
FAILURE TO NEUTRALIZE THE POLIOMYELITIS VIRUS WITH SERA OF ADULT MACACUS RHESUS AND OF YOUNG FEMALE RHESUS TREATED WITH ANTERIOR PITUITARY EXTRACTS*

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The usual conception of the epidemiology of poliomyelitis is that man is relatively resistant to the disease, that the virus is commonly transmitted from and to the nasopharynx and is more widely distributed than is evidenced by clinical records, and that the general population is largely immunized by these factors of host resistance and parasite distribution. Difficulty of direct experimental proof of these principles lies in the fact that the virus is not cultivable by bacteriological methods and hence is dependent upon animal inoculation for its demonstration. By the use of this method, however, the virus has been recovered in non-clinical cases in a few instances (1). Several more indirect types of evidence are available to support the classic conception, but the one we are here concerned with is the power of the serum of certain adults without history of poliomyelitis to neutralize the virus, a property which has been assumed to be due, by analogy with other diseases, to subclinical infections with the causative agent.

The view that such an assumption is not entirely tenable has been expressed by Jungeblut and Engle, who suggest that "resistance to poliomyelitis .... is predominantly a function of normal physiological maturation and to a large extent seems to develop independently of previous contact with the specific antigen" (2a). This

theory is based on their ability to demonstrate the neutralization of virus with the sera of certain adult *Macacus rhesus* and of immature female *rhesus* caused to menstruate by injections of anterior pituitary extracts (2 *a*, *b*). These monkeys had had no exposure to poliomyelitis virus, and hence the occasional neutralization was attributed to endocrine factors and maturation. More recently, the same authors have detailed the experiments with adult monkeys of both sexes and with young females treated with a variety of gonad-stimulating principles. They report (2 *c*) that a certain proportion of each group was shown to possess neutralizing serum and some were resistant to infection by the cerebral route.

We have been concerned with this problem of specificity in poliomyelitis immunity and have attempted to obtain experimental data in support of the theory of physiological ripening as a factor in resistance to this disease. The present communication reports the results of tests on the sera of adult *rhesus* monkeys and of young female monkeys menstruating after treatment with extracts of the anterior pituitary. The technic of testing the serum was as previously described (3), employing 3 parts of serum to 1 part of 1.25 per cent centrifugated monkey virus cord (PMV strain). Monkeys fatally injected with these mixtures were diagnosed as dying of experimental poliomyelitis on the bases of fever, paralysis, and histopathology of the cord. Virus and serum control monkeys were included in the several experiments, each of the former dying of poliomyelitis and each of the latter surviving without symptoms.

EXPERIMENTAL

The first neutralization tests were carried out on the sera of adult male and female monkeys. These animals were judged to be mature or submature on the basis of their height, weight, and dental and sexual development. The dentition was either completely permanent or early permanent in type. The males possessed completely descended testicles and the females were passing through more or less regular, but definite, menstrual cycles. Most of these animals had been in the department animal quarters for a year or more (prior to the first test) and were used for various bacteriological purposes; the majority of them had been injected with mixtures of serum and poliomyelitis virus but had never shown any evidence of this disease.

TABLE I

Tests of Serum of Adult Male Monkeys for Property of Neutralizing Poliomyelitis Virus

Monkey	Description				Dates bled	Remarks	Serum neutralization of poliomyelitis virus
	Weight	Height*	Dentition	Sexual development			
	kg.	cm.					
A	4.1	67	Early permanent	Testes fully descended	June 9, 1932	These animals had received human nervous tissue 6 mos. before test	Negative
B	4.9	71	" "	" "	Mar. 22, 1933		Negative
C	5.0	73	Complete permanent	" "	Mar. 22, 1933		Negative
D	6.5	74	" "	" "	June 9, 1932	These animals had received an intracerebral injection of a serum-virus mixture nearly 2 yrs. before first bleeding; have been used in studies on intestinal flora for the last 3 yrs.	Negative
	7.8	84			Mar. 30, 1933		"
E	6.2	71	" "	" "	June 9, 1932		Negative
	7.6	84			Mar. 30, 1933		"
F	6.8	72	" "	" "	June 9, 1932		Negative
	7.3	82			Mar. 30, 1933		"
G	5.6	74	" "	" "	June 9, 1932	Had been kept with fatally injected poliomyelitis monkeys for 15 mos.	Negative
H	5.3	72	" "	" "	June 7, 1932	Normal monkey	Negative
I	8.3	Not determined	" "	" "	Apr. 18, 1933	Father of male born Mar. 17, 1933	Negative
J	5.0	71	" "	" "	June 9, 1932	Immunized by cutaneous inoculation of virus cord 1 yr. before	Positive

* Measured from crown to heel.

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Tests of Serum of Adult Female Monkeys for Property of Neutralizing Poliomyelitis Virus

TABLE II

Monkey	Weight kg.	Height• cm.	Dentition	Description — Menstrual state at time of taking serum for test	Dates bled	Remarks	Serum neutralization of poliomyelitis virus
K	4.9	70	Complete permanent	Specimen taken 2nd day of vaginal bleeding	June 9, 1932	Had been kept with fatally injected poliomyelitis monkeys for 15 mos.	Negative
			"	Edema and hyperemia of sex skin; no gross vaginal bleeding	Jan. 18, 1933		"
			"	No edema or hyperemia of sex skin; no gross vaginal bleeding	Mar. 8, 1933		"
L	6.0	Not determined			Apr. 18, 1933	Mother of male born Mar. 17, 1933; bled 32 days postpartum	First test positive. Second test negative (undiluted and diluted 1:5)
M	3.2	63	Early permanent	Specimen taken 9 days after onset of menstruation	Mar. 18, 1933		Negative

N						Repeatedly inoculated cutaneously with normal monkey cord 1 yr. before	Negative
3.7	68	Early permanent	Specimen taken 10 days before onset of menstruation	June 9, 1932		Negative
			Edema and hyperemia of sex skin; vaginal bleeding 6 days after specimen taken	Jan. 18, 1933		"
			Faint hyperemia of sex skin; specimen taken 14 days after onset of menstruation	Feb. 7, 1933		"
			Faint hyperemia of sex skin; vaginal bleeding 8 days after specimen taken	Feb. 28, 1933		"
			Slight hyperemia of sex skin; specimen taken 1st day of vaginal bleeding	Mar. 8, 1933		"
			Faint hyperemia of sex skin; specimen taken 9 days after onset of menstruation	Mar. 17, 1933		"

* Measured from crown to heel.

Twelve samples of undiluted serum from nine adult males were tested. Six of these specimens were obtained in June, 1932, and were uniformly devoid of neutralizing power. Since it was proposed by Jungeblut (4) that a seasonal factor might enter the problem, three of the animals still available were bled again in March, 1933. Serum was also obtained at this time from three other adult males. These six undiluted sera likewise failed to inactivate the virus (for details of experiment, see Table I). The serum of a tenth adult male immunized intracutaneously with virus cord, served as a control in this and subsequent experiments and invariably neutralized the virus.

A similar lack of virucidal property was observed in the sera of four adult females. Of the eleven specimens tested, two were obtained in June, 1932, the others from January to April, 1933. Ten samples, drawn before, during, or after menstruation, failed completely to neutralize the virus when used undiluted. The eleventh sample, taken from a female 32 days postpartum, gave equivocal results: in the first test, it inactivated the virus; but when titration was attempted with the serum undiluted and diluted 1:5, it failed to neutralize in both concentrations (Table II).

These experiments did not reveal a correlation between the virucidal capacity of monkey serum and maturity, with the possible exception of the instance just mentioned. Furthermore, physiological fluctuations in endocrine balance, as exemplified by the menstrual cycle, failed to induce the appearance of virus-neutralizing substances in the serum of the animals tested. The opportunity of determining the resistance of the adult monkeys to infection by cerebral injection was not afforded.

We attempted to confirm Jungeblut and Engle's observation that immature monkeys treated with a gonad-stimulating principle of the anterior pituitary occasionally yielded serum possessing virucidal properties (2 b, c). Our experimental animals for this purpose were young female *rhesus* monkeys weighing between 2 and 2.5 kilos and possessing entirely deciduous teeth. Their sexual development appeared to be in a completely immature state.

The gonad-stimulating substance (5) used in these experiments was a clinical preparation made by Mrs. Zonja Wallen-Lawrence of the Department of Physiological Chemistry and Pharmacology. It was

prepared from whole sheep pituitary powder by a method which will be reported from that laboratory. Anterior pituitary extract was administered to ten monkeys intramuscularly in equal daily amounts (except in two instances) over periods varying from 5 to 13 days. As treatment progressed the area in the region of the buttocks, called the sex skin, became hyperemic and edematous. By means of rectal

TABLE III

Tests of Serum of Young Female Monkeys Treated with Anterior Pituitary Extracts for Property Neutralizing Poliomyelitis Virus

Monkey	Description*		Anterior pituitary extract injected	Number of injections intramuscularly	Dates of menstruation‡ during period of observation	Serum specimen taken after onset of menstruation	Serum neutralization of poliomyelitis virus
	Weight	Height†					
	kg.	cm.	1933		1933	days	
1	2.0	55	Feb. 14–26	13	Mar. 3–12	9	Negative
			Apr. 19–28	10	Apr. 30–May 4	9	"
2	2.1	58	Feb. 14–26	13	Mar. 1–20	11	"
			Apr. 19–28	10	Apr. 30–May 5	9	"
3	2.4	60	Feb. 14–26	13	Mar. 2–11	9	"
			—	—	Apr. 24–May 4	10	"
			—	—	May 21–25	10	"
4	2.2	57	Feb. 14–26	13	Feb. 26–Mar. 9	9	"
5	1.9	57	" 14–26	13	" 26– " 9	9	"
6	2.0	56	May 16–19	4	May 29–June 1	7	"
7	2.2	53	" 16–20	5	" 27–31	9	"
8	2.2	56	" 22–28	7	June 3–6	9	"
9	2.3	55	" 22–28	7	" 3–6	9	"
10	2.0	56	" 22–31	10	" 13–16	8	"

* Completely deciduous dentition in all monkeys of this group.
† Measured from crown to heel.
‡ As determined by microscopic examination of vaginal washings.

examination, an increase in the size of the ovaries and the uterus was noted, while vaginal lavages, which during a control period prior to treatment were negative, revealed mucus and a rapidly increasing number of leucocytes and epithelial cells. Upon cessation of treatment the intensity of the hyperemia and edema in the sex skin diminished and gross uterine bleeding occurred. The ovaries and uterus also decreased in size at this time.

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Blood for the neutralization tests was drawn from 7 to 11 days after the onset of uterine bleeding, in conformity with the interval used by Jungeblut and Engle (2 a). Fourteen specimens of serum from the ten females were tested undiluted. Nine of these animals received intracerebrally a mixture of their own serum plus virus. The serum of the tenth female, which succumbed to intercurrent infection in the interim between bleeding and the test, was examined in an unused monkey (as were the remaining four specimens). None of the fourteen sera neutralized the virus (Table III).

At the conclusion of the experiment the sexual tract of each monkey was studied. The ovaries were juvenile in appearance and in none was there any evidence of a corpus luteum, nor was there any other indication of ovulation. In every case the uterus appeared un-developed and the mucous membranes presented the condition typical of puberty. The uterine mucous membrane was 1 mm. or less in thickness in all the animals except Monkey 3, in which it was 2 mm. thick. There was no sign of pseudopregnancy anywhere in the uterine mucosa. Nothing resembling sexual maturity was found in any of these animals.

From these findings—the hyperemia and edema of the sex skin followed by a menses-like bleeding which occurs normally in *M. rhesus* at the time of puberty, together with the degree of development in the sexual organs—it is concluded that the administration of anterior pituitary extract caused in our monkeys a precocious sexual develop-ment to a state similar to that found at puberty, but which cannot be interpreted as sexual maturity. Such a condition induced neither a virucidal capacity of the serum nor a systemic resistance to the virus of poliomyelitis.

COMMENT

The technic of the neutralization test employed in these experiments is somewhat different from that generally used elsewhere, in that the serum-virus ratio is 3:1 and the amount of virus is reduced to the supernatant of a centrifugalized 1.25 per cent emulsion of monkey virus cord. While this technic may seem to favor the determination of serum virucidal property, our results in examining human sera have been entirely comparable to those of other laboratories. In fact,

the results of our testing Chinese sera (6) were the same as those obtained by Jungeblut (7) in examining specimens of identical origin and lot. Our dose of virus, on the other hand, is well above the minimal infective dose, so that no false positive results have occurred. The significance of the negative serum tests reported in this paper is more apparent, in view of these remarks on technic.

The only possible evidence in these experiments for the influence of a physiological factor in resistance to the experimental disease was the result of testing the serum of an adult female 32 days postpartum. The same specimen neutralized the virus in one test but not in a second, which may be ascribed either to a low virucidal content perhaps referable to the previous pregnancy or to a peculiar resistance on the part of the young test monkey.

It is interesting to note that two adult monkeys, male and female, included in these series, did not acquire a serum virucidal power through intimate contact over a period of 15 months as cage mates with numerous monkeys succumbing to injections of virus.

The possibility that a general bodily resistance to infection with poliomyelitis virus might have been produced in the immature animals treated with anterior pituitary extract was taken into consideration. The serum-virus mixtures were injected into the brain of the animal that had furnished the serum, so that factors other than the possible virucidal capacity of the serum might exert their influence. In this group, too, every monkey succumbed to infection.

Our inability to effect neutralization with the sera of adult male and female monkeys and of young monkeys induced to menstruate by artificial means lends experimental evidence to the view that the virucidal property of serum is not attributable only to maturity or to certain fluctuations in the physiological state of the individual.

SUMMARY AND CONCLUSIONS

1. Twelve specimens of serum from nine adult male monkeys failed to neutralize the virus of poliomyelitis.

2. Ten samples of serum obtained from three adult female monkeys at various phases of the menstrual cycle likewise proved incapable of neutralizing the virus. An eleventh serum, drawn from a fourth female 32 days postpartum, gave irregular results. It neutralized

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once and failed to do so on second test. This is the only suggestion in our experiments that a physiological factor may play a part in poliomyelitis immunity.

3. Fourteen sera from ten immature monkeys caused to menstruate by treatment with anterior pituitary extract were devoid of virucidal property. This treatment failed also to induce a systemic resistance to intracerebral injections of virus in the nine monkeys of the same group available for test.

4. We were unable to demonstrate in our monkeys a correlation between virucidal capacity of the serum and maturity or physiological variations as exemplified by menstruation.

We wish to thank Mrs. Zonja Wallen-Lawrence for the anterior pituitary extracts, Dr. I. Schour of the University of Illinois College of Dentistry for the determination of dentition, and Dr. George W. Bartelmez of the University of Chicago for the interpretation of the generative tract findings in the young treated monkeys.

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STUDIES ON MENINGOCOCCUS INFECTION

VI. The Carrier Problem

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Cerebrospinal meningitis was amongst the first diseases in which the importance of the carrier problem was recognized, and probably in no disease has this problem received more attention. Following on similar discoveries in connection with cholera by Koch (1) and diphtheria by Escherich (2), Kiefer (3) and Albrecht and Ghon (4) showed that the organism could be recovered from the nasopharynx of many patients with meningitis and also from apparently healthy individuals who had been in contact with cases. These facts were shortly thereafter confirmed by numerous investigators among whom von Lingelsheim (5) was preeminent. Although the fact was generally taken for granted, it remained for English investigators (6) during the war time epidemic to show conclusively that in the majority of cases the organisms found in the throats are of the same type as those isolated from the spinal theca and are undoubtedly the identical strains. These demonstrations drew the attention of investigators to the nasopharynx both as the presumptive primary area of infection and as the possible focus from which the disease can be transferred to other individuals. Carrier transmission was believed to be more important than case to case infection in view of the extreme rarity of obvious infection from case contact (7, 8) and further from the finding of von Lingelsheim (5) that the majority of cases of cerebrospinal fever gave negative throat cultures after 3 weeks.

The fact that meningococci could be demonstrated in the nasopharynx of apparently healthy contacts (3, 4) was, of course, of the utmost importance. These carriers might acquire the nasopharyngeal infection and transmit it without themselves showing any symptoms beyond possibly those of a slight pharyngitis. In the course of several such transmissions the meningococcus might fall on suitable soil and be capable of invading the body beyond the mucous membranes to produce a general infection and meningitis.

Kutscher in 1906 (9) showed that carriers existed not only amongst those who had been in contact with accepted cases of the disease but also amongst the general population. This has been confirmed by many others, and further it has been shown, as was only to be expected, that the carrier rate is highest amongst close contacts and lowest amongst non-contacts, with secondary contacts occupying an intermediate position.

There has been much discussion as to whether the meningococcus can be looked upon as a relatively normal inhabitant of the nasopharynx like the pneumococcus and certain other pathogens, or whether its occurrence there is abnormal. The weight of opinion has been in favor of the latter and in support of this the short duration of nasopharyngeal contamination is cited amongst other facts. Perhaps, however, opinion has been prejudiced by the varying ideas as to what is to be understood by the name "meningococcus." This point is discussed below but it can be said here that the confining of "meningococcus" to strains which correspond in every way, i.e. even serologically, with those strains isolated from the spinal fluid of the majority of cases, would tend to limit the problem and to give more truth to the statement that the meningococcus is of abnormal occurrence in the nasopharynx.

Another point in favor of regarding the meningococcus as an abnormal inhabitant of the nasopharynx is the claim that the presence of the organism is usually, if not invariably, associated with an inflammatory condition of the mucosa (10). However, the accuracy of this claim has been questioned by several other workers (11, 12) and it seems doubtful whether an association with inflammation, other than one which is seasonal and fortuitous, exists, though it is true that there have been reports of an epidemic of meningococcal nasopharyngitis which has preceded or run concurrently with the epidemic of meningitis.

Bruns and Hohn (13) demonstrated a close relationship between the carrier rate in the general population and the onset, rise and decline of an epidemic. Glover (14) had special opportunities to make observations upon this point during war time troop concentration and he noted that marked overcrowding of individuals produced a rise in the carrier rate. When such a rise exceeded a certain figure, which Glover put at 20 per cent, the community was in grave danger of an outbreak of cerebrospinal fever. An epidemic of carriers always preceded the epidemic of meningitis. Glover was also able to show that the increase in carrier rate was directly dependent upon such factors as overcrowding, deficient ventilation and cold, damp weather. Such rises occurred most strikingly amongst groups of young recruits who presumably presented the additional factor of a favorable soil. It is quite possible that the meteorological factors acted merely by increasing the overcrowding and deficient ventilation, for the men crowded together for warmth and company on wintry evenings.

Many attempts have been made to cure carriers so that the risk of an epidemic could be lessened. The methods adopted have varied from simple isolation and observation to gargling or intensive saturation of the mucous membrane with germicides such as protargol, chloramine-T or zinc sulfate (15), and even to removal of the tonsils and adenoids in those infections which were so stubborn that they resisted the more conservative treatments. Each of the methods was adjudged by means of weekly or biweekly throat swabs, and three successive negative cultures were held to constitute a cure of the carrier condition. It is doubtful whether the results obtained by gargling or intensive vapor inhalations were much better than those from simple isolation. It has been the opinion of

the majority of investigators that by far the larger number of carriers have cleared up spontaneously at the end of the 4th week and three successive throat cultures at this time have proved negative (5, 16, 17). Moreover, in the large proportion of cases it is held that the cure is permanent and that the meningococci do not recur (von Lingelsheim (5)). However, von Lingelsheim also points out that the organisms may reappear after a 3 or 4 week negative interval, and single or scattered examples of such recurrence have been reported (17). Unfortunately there have been relatively few reports of careful subsequent examination of "cured" carriers, and one can obtain no clear idea from the literature how often the reported absence of recurrence is due to lack of such examination.

It has been stated that the persistence of a strain in the nasopharynx is an abnormal event and depends upon abnormalities of the upper respiratory tract (18–20). Such abnormalities could be treated surgically and chronic carriers would clear up. Here again, however, it must be pointed out that the criterion of cure was that of a few consecutive negative nasopharyngeal cultures and that no later examinations of these cases were undertaken.

The certain identification of meningococci in the culture from the nasopharynx has been a matter of much controversy. Although Albrecht and Ghon (4) in 1901 had described the preparation of agglutinating serum in rabbits, it was not until later that the agglutination test, as the result of work by Flexner (21), Kolle and Wassermann (22) and others, was generally adopted. Before then the diagnosis was somewhat uncertain and depended in the main on colony morphology, staining properties and fermentation reactions (23), all of which had been carefully worked out by von Lingelsheim (5). Once antimeningococcal serum had been prepared, however, the agglutination reaction was made the criterion of genuineness, only to be rendered doubtful in turn by the subsequent work of Dopter (24). He was able to isolate from the nasopharynx an organism which had all the traits of the meningococcus but did not agglutinate in a serum prepared with strains at that time occurring in the spinal fluid of cases of meningitis. Dopter would not admit that this organism was not a meningococcus and proposed for it the name "parameningococcus." His view was confirmed by Carnot and Marie (25) who succeeded in isolating this type (II and IV by the later Gordon classification) from cases of meningitis during the following year.

It is now recognized that all four of Gordon's cerebrospinal meningitis types can be found in the nasopharynx of healthy individuals, both those who have and those who have not been exposed to the disease; further, that in epidemic periods the proportion of these types in the throats of contacts and, to a lesser degree, in those of the general population shows a remarkable correspondence with the distribution of types among the frank cases of meningitis (12, 16, 17). Certain strains of Gram-negative cocci from the nasopharynx are, however, still the subject of argument. While the majority of Gram-negative cocci can readily be distinguished from meningococci and placed in their appropriate groups by means of the carbohydrate fermentation test, colony characteristics and pigment formation, yet there still remain strains which behave in every way as true meningo-

cocci save that they are not agglutinated by any of the four Gordon sera or by polyvalent serum. In the past, the weight of opinion has been against the supposition that such strains are true meningococci. They were called "pseudo-meningococci" or "pharyngococci" (12, 26, 27) and their presence in the naso-pharynx was ignored in the estimation of the meningococcus carrier rate. Yet Eastwood (28) and Griffith (29) claimed that these "pseudomeningococci" had every right to be considered as true meningococci, and Scott (30) was able to show that although the strains failed to agglutinate with any of the monovalent type sera prepared with spinal fluid strains, yet they were closely allied to the four Gordon types and could absorb agglutinins from the type sera. The majority of atypical strains which Scott examined were found to be allied to Type II.

In conclusion of this summary, it may be pointed out that the previous work has served to emphasize the importance of the carrier in the spread of cerebrospinal meningitis and the necessity for naso-pharyngeal examination of contacts and others in order to establish the carrier rate and to be forewarned of the danger of an epidemic. Newsholme (31) has estimated that during the course of an epidemic of a year's duration nearly everyone within the epidemic zone will harbor the meningococcus at some time. The general opinion, based on the assumption that three consecutive negative swabs constitute a cure, has been that meningococcus carriers, unlike those who carry for example *Corynebacterium diphtheriae*, are contaminated for only a short time and that while radical methods assist in clearing up the condition especially if it be stubborn, yet the majority of cases undergo spontaneous and permanent relief, leaving only a very small number of chronic carriers. It is also believed by many that meningococci are to be regarded as abnormal inhabitants of the nasopharynx and further that the strains from the throat can be identified with Gordon's four types. Other strains, which fulfill all the requirements for the biological characteristics of the meningococcus save that of agglutina-tion in monovalent or polyvalent antimeningococcal sera, are excluded by most investigators from the group of true meningococci.

A consideration of the results of other investigators as outlined above has led to the work which is presented here. Of particular interest was the question of the duration of the carrier state in the absence of any treatment. Was it true that the period of contamina-tion was limited to 3 or 4 weeks? If not, how long a period of apparent remission could occur and usually did occur? In the case of reappear-

ance of the carrier state, should that take place, was the organism the same as that which had formerly been isolated on weekly swabbings, or had there been a change of type or of minor characteristics? Furthermore, it seemed of interest to examine the distribution of types amongst carriers at this non-epidemic time and to compare it with other studies of carrier types made during epidemic and non-epidemic periods. Finally an attempt was to be made to trace the relationship, if any, of the carrier state and its periods of waxing and waning to diseases of the upper respiratory tract.

A partial answer to some of these questions has arisen out of the present work and will appear as the results are outlined below. It is believed that a study undertaken during an epidemic period might serve to enlighten the problem still more.

Material and Technique

Investigations were carried out on three groups of normal individuals which, none of the members having been known to be in contact with cases of cerebrospinal fever, could be considered as non-contact groups.

The first group consisted of all the individuals working on one floor of a building unit of The Rockefeller Institute in New York City. This group of adults included secretaries, technicians and research investigators, both male and female —in all 24 persons. Of these 24 only three had much laboratory contact with strains of meningococcus owing to the work in which they were engaged. The second group consisted of 25 girls between the ages of 6 months and 14 years, who were inmates of a foundling home and were confined to an isolation unit on account of gonococcal vaginitis.[1] The last group consisted of 569 young men from 18 to 25 years of age drawn chiefly from the less fortunate sections of the community and collected in a concentration camp at Fort Slocum preparatory to sending them out to forestry camps.[2] Since the investigation of each of these three groups differed considerably, owing to uncontrollable factors, each group will be discussed separately.

Both straight, unprotected swabs and those on curved wires enclosed in a protecting West tube made of glass were used, and care was taken to obtain

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[1] The opportunity is taken to thank Dr. Joseph A. Dillon and all of the staff of the New York Foundling Hospital for their kind cooperation.

[2] The author wishes to express his gratitude to Colonel C. R. Reynolds of the Second Corps Area Headquarters and the late Colonel George P. Peed, surgeon at Fort Slocum, whose kind cooperation made this work possible.

material on the swab only from the nasopharynx behind the soft palate. It was found that experience with the straight swab allowed one to obtain just as good results as with the West tube. Any swab touching the tongue or tonsils or becoming smeared with saliva was discarded for it was found, in complete corroboration of the work of Gordon and others (32, 33) that contamination with salivary bacteria or their products will inhibit the growth of the meningococcus.

The nasopharyngeal swab was smeared on a large Petri dish, 14 cm. in diameter, freshly poured with pneumococcus agar containing freshly drawn citrated rabbit blood and 0.03 per cent dextrose.[3] By using a large plate and changing to a sterile swab for the spreading of the material after the initial smear with the infected swab had been made, good separation of colonies was effected and picking of questionable colonies facilitated. It is important to have the agar plate warmed to 37°C. when the plating is done, otherwise the growth of the meningococcus may be partially or completely inhibited. Directly after spreading, the plate is returned to the 37°C. incubator and left overnight. The next morning it is examined and parts of suspicious colonies are smeared on a glass slide, stained by Gram's method and examined. Those colonies which prove to be Gram-negative cocci are transferred to freshly poured blood agar plates and an estimate is made of the percentage of that particular type of colony on the plate. This transfer in turn grows out overnight and if it proves the next day to be a pure culture, its fermentation and agglutination reactions are tested. It has been found that more rapid and satisfactory results of the fermentation reaction are obtained if solid rather than fluid media are used.[4] All agglutination reactions are carried out at 37°C. for 2 hours and overnight in the ice box (35).

The Institute Group

The investigations on the first group—those at The Rockefeller Institute—were the most thorough. All of the individuals examined worked on one floor of one of the building units of the Institute and

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[3] Fresh beef heart (free from fat and gristle)................. 500 gm.

    Witte peptone... 10 "

    Sodium chloride... 5 "

    Agar.. 20 "

    Dextrose.. 0.3 "

    Tap water... 1,000 cc.
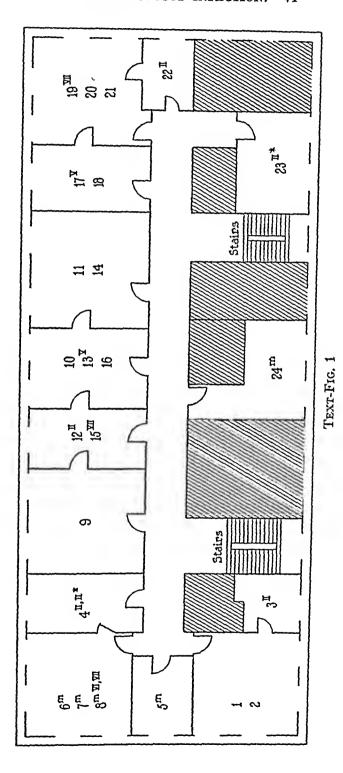
10 cc. of citrated rabbit blood are added to each 250 cc. of medium.

[4] Beef heart agar, as given in footnote 3 but without the dextrose, is used. To 90 cc. of this agar, melted and cooled to 45°C., is added 10 cc. of a 10 per cent solution of the sugar to be tested. The sugar solution, made up in distilled water, has previously been sterilized by heating at 10 pounds steam pressure for 12 minutes. To 100 cc. of the sugar-agar are added 5 cc. of hemolyzed blood and 2 cc. of Andrade's indicator (standard) (34).
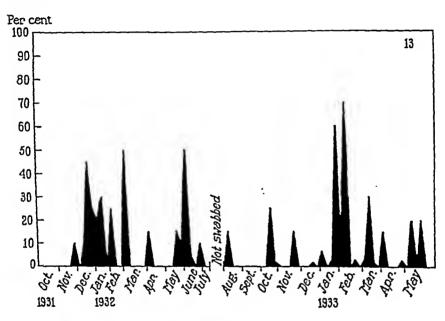
a plan of this floor is shown in Text-fig. 1. A certain number of these individuals were engaged in purely secretarial work, but the majority occupied themselves in active research upon pathological and bacteriological subjects. Each individual is indicated in the room in which he, or she, worked by a number. If the individual was a carrier, the strain type is indicated. Those working with meningococci are shown by means of a small *m*. During a period of 20 months, 24 persons were subjected to nasopharyngeal swabbings which were carried out approximately weekly. Of these 24, thirteen were observed throughout the full 20 months. The other eleven, who arrived or departed during the course of the investigation, were observed over shorter periods; that is to say, two for 15 months, eight for 9 months and one for 7 months. Ten individuals carried meningococci in their nasopharynx at some time or other during the period of observation and these ten carriers can be divided into three groups. In the first group are five individuals who were constant or chronic carriers; in the second, two who were intermittent carriers; and in the third, three who were transient carriers. These three groups will be dealt with in detail. It is important to note that only one of these carriers—No. 8—came in contact with meningococcus research work in any way. Two non-carriers handled meningococcus cultures, on solid or on fluid media, constantly and two more handled such material occasionally. These four workers—Nos. 5, 6, 7, 24—remained free from the meningococcus throughout the period of investigation, although one—No. 5—twice accidentally received about 2 cc. of living and presumably pathogenic meningococcus broth culture into his mouth.

Three of the five chronic carriers were males and two females. The latter individuals were engaged in secretarial work. Protocols of these five individuals follow.

No. 13.—(Text-fig. 2.) Adult male. This individual was negative when first swabbed, Oct. 6, 1931. He became positive on Nov. 24, showing meningococcus colonies for about 10 per cent of his throat plate. From then onwards he was a more or less constant carrier until the end of the investigation on May 21, 1933, when his throat plate showed 20 per cent of meningococci. In all he showed 33 positive cultures out of 82 swabbings. In between these two dates he had intervals of freedom from demonstrable infection. These varied from a single negative swabbing up to 8 consecutive negatives or a free period of 2 months. On the whole,

TEXT-FIG. 1

the numbers of meningococci were not high but on one occasion—Feb. 7, 1933— the plate showed some 70 per cent of meningococcus colonies. There was some tendency for the meningococci to be more plentiful in the late winter and spring, *i.e.* January to April, and the longest free interval occurred in the late summer and early autumn, August to October, but the seasonal variation was not particularly marked with this individual. The same type was found throughout—a Type V according to the terminology adopted in this laboratory. As will be pointed out below, this type belongs to the Group I, that is to say, is allied to Type I-III.
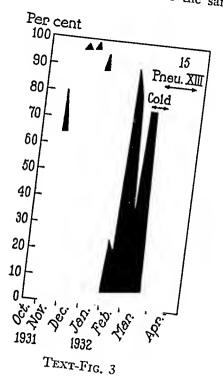
TEXT-FIG. 2

Individual 13 was swabbed again on three occasions in Nov., 1933. On one of these he was positive and showed the same Type V as he had carried previously. Thus he is known to have been a constant carrier of the same type of meningococcus for at least 24 months.

No. 15.—(Text-fig. 3.) Adult male. This individual was negative when first swabbed on Oct. 6, 1931, but became positive on Nov. 3, with meningococci forming 80 per cent of his throat plate. From then on he remained constantly positive until Mar. 22 at which time he was recovering from a cold, as will be pointed out below. He showed almost throughout the period of investigation surprisingly large numbers of meningococci on his throat plates, ten plates showing 90 per cent or over and two being apparently pure cultures. In all, 22 out of 29 swabbings were

positive. On Mar. 10, 1932, he developed a severe coryza. At this time his plate showed 75 per cent meningococci. 5 days later the numbers of meningococci had fallen to 5 per cent and the rest of the colonies were all a pneumococcus which proved to be Type XIII. A week later the meningococci had disappeared and the Pneumococcus XIII was still predominant. Up to the end of April, that is for 6 weeks, the Pneumococcus XIII persisted and meningococci did not reappear. At the end of April this individual unfortunately left the city and could be followed no longer; but two single reports on throat cultures state that he remains free from meningococci. The type carried was the same throughout: namely,

Per cent

TEXT-FIG. 3
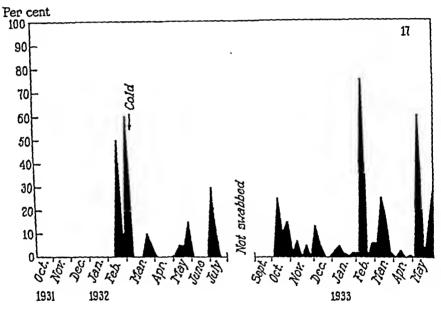
a Type VII which, as will be pointed out below, belongs to the Group II and is thus related to Type II.

No. 17.—(Text-fig. 4.) Adult male. This individual was negative from Oct. 6, 1931, until Feb. 15, 1932. On this date he showed meningococci in 50 per cent of his throat plate. From then on he was a more or less constant carrier, giving 36 positive cultures out of 63 swabbings. Free intervals were not infrequent and varied from a single swabbing up to three consecutive negatives twice, four consecutive negatives once and five consecutive negatives once. The infection was not heavy but reached as high as 75 per cent on one occasion. Here again there was some tendency for the meningococci to be most plentiful in the late winter and spring and for the smallest numbers and longest free intervals to be found in the late

summer and autumn. The strain proved to be a Type V and remained unchanged during the period of investigation.

This individual was swabbed again three times during Nov., 1933. On one occasion he showed a meningococcus on his throat plate, of the same type as carried previously—a Type V. He is thus known to have been a carrier for 21 months.

No. 22.—(Text-fig. 5.) Adult female. Secretary. This individual was positive when first swabbed, Oct. 6, 1931, and remained a constant carrier from then on for the remainder of the investigation. In all, 75 out of 92 cultures were
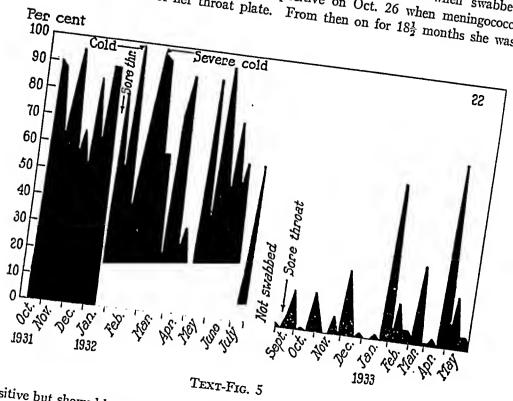
TEXT-FIG. 4

positive. Intervals of freedom from meningococci were usually only for a single swabbing, but twice there were two consecutive negatives. The numbers of organisms as shown by colonies on the plate were usually high and on nine occasions reached 90 per cent or over. The percentage was consistently higher in this individual than in any other. The contamination showed a decided tendency to be less in the autumn and early winter in this patient, as is shown by Text-fig. 5. Periods of coryza did not affect the numbers of organisms, but on two occasions pharyngitis, from which slightly increased numbers of non-hemolytic streptococci could be isolated, was associated with a disappearance of meningococci. The strain was throughout a typical Type II indistinguishable from spinal fluid Type II strains from cases of meningitis.

The individual was swabbed again during Nov., 1933. On each occasion she was positive with figures of 50, 75 and 30 per cent. The strains were still typical Type II. She is thus known to have been a carrier of Type II for at least 26 months.
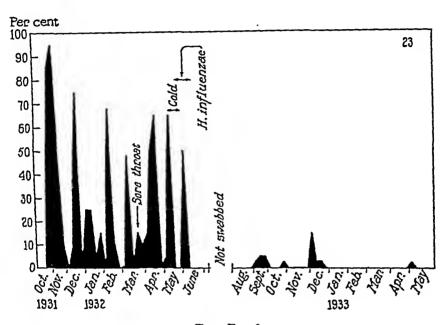
No. 23.—(Text-fig. 6.) Adult female. Secretary. Negative when swabbed on Oct. 6, 1931. She was first found positive on Oct. 26 when meningococci formed 85 per cent of her throat plate. From then on for $18\frac{1}{2}$ months she was

TEXT-FIG. 5

positive but showed long intervals of freedom in one case amounting to $4\frac{1}{2}$ months or 18 consecutive negative swabbings. In all, 40 out of 88 swabbings were positive. The numbers of organisms were moderately high and twice reached 90 per cent or over. There was no definite seasonal variation in this individual. However, in common with all the other chronic carriers she showed fewer meningococci in the year 1932–33 than in 1931–32 when the investigation was commenced. The type remained the same throughout; namely, an atypical Type II of which more will be said below. For the first 7 months this individual was almost constantly positive. In May of 1932 she had a severe cold which was followed by an outgrowth of H. influenzae in almost pure culture. Subsequent to this the meningococci disappeared for $3\frac{1}{2}$ months and on reappearance were in small numbers and present only irregularly. Here again, as with No. 15, the appearance in the nasopharynx of a pathogen in almost pure culture was associated

with a great diminution in the numbers of meningococci. In the present case, however, there was no permanent relief.

This individual was swabbed again on three occasions during Nov., 1933. On each occasion she was positive, the meningococci being present in small numbers—5, 2 and 3 per cent. The type was the same as before; namely, atypical Type II. Thus she is known to have been a carrier of an atypical Type II for at least 25 months.
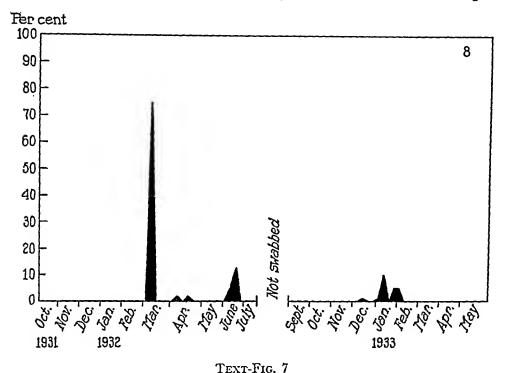
TEXT-FIG. 6

Four of these five individuals have thus been known to be chronic carriers for periods ranging from 21 to 26 months, and they show no signs at the present time of undergoing spontaneous relief. In the only case in which there was apparently relief, it followed the appearance in the nasopharynx of another pathogen, namely a Type XIII pneumococcus, in great abundance. In every case the type of the organisms involved remained the same throughout and certain small peculiarities in the individual strains which were characteristic, *i.e.* ease of emulsion and the like, made it certain that in each instance the same strain persisted throughout.

Both of the intermittent carriers were males. One of them, No. 8,

was engaged in the meningococcus research work as technical assistant. Although there were five individuals in all who came into some contact with the meningococcus research work, he was the only one who developed a nasopharyngeal contamination. Protocols follow.

No. 4.—Adult male. This individual was positive only twice in the course of 9 months (34 swabbings). On the first occasion the strain was a typical Type II and appeared in 10 per cent of the throat plate. 3 months later he was again

TEXT-FIG. 7

positive, the strain this time being an atypical Type II which appeared in 3 per cent of the throat plate. Three subsequent swabbings during Nov., 1933, were all negative.

No. 8.—(Text-fig. 7.) Adult male. This individual was negative for 5 months and then in Mar., 1932, became positive with meningococci forming 75 per cent of his throat plate. During the next 15 months he was positive on nine additional occasions, the numbers of organisms usually being small. There were long intervals of freedom amounting in one instance to 6 months and in another to 4 months. There was a definite winter and early spring incidence. Although all the cultures isolated belonged to Group II, there were individual variations between different cultures. The majority of cultures were classed as Type VII but one at least was Type VI, and it seems certain that this individual did not

harbor the same strain throughout the period of observation. The possibility that this individual was infected by the cultures he handled would seem to be ruled out by the fact that at no time has a strain similar to the Type VI strain been seen in the laboratory.

He was swabbed again on three occasions during Nov., 1933, and on one of these showed 2 per cent meningococci on his throat plate. This strain belonged to Group II.

In neither of the intermittent carriers, therefore, did the strain remain the same throughout the investigation. It may be that one was dealing with the same strain throughout but that it was undergoing definite variations in antigenicity and other characteristics. In favor of this is the fact that in each case the different strains one and all belonged to Group II. Yet the individual variations were striking and, in view of the long intervals of freedom, it is perhaps easier to suppose that one is dealing with examples of spontaneous cure and reinfection in persons perhaps peculiarly susceptible to the carrier state.

Nos. 3, 12 and 19.—The three transient carriers were all adult males. In each case the meningococcus appeared in the throat on one occasion only. In No. 3 the infection formed 40 per cent of the plate, but in Nos. 12 and 19 the infection was only slight, being 3 per cent and 5 per cent respectively. All the strains belonged to Group II, those of Nos. 3 and 12 being Type II while that of No. 19 was a Type VII. They were observed for 20, 9 and 15 months respectively. Reswabbing in Nov., 1933, gave negative results.

The investigation of this group of 24 individuals brings out certain points of interest. In the first place, it can be stated that this is apparently the first time that any group of individuals, including carriers of the meningococcus, has been subjected to such intensive and prolonged investigation. Of the ten carriers, six were observed for 20 consecutive months, one for 15 months, two for 9 months and one for 7 months. In every case, except No. 15, there was an unavoidable gap of between 2 and 5 weeks in the summer when vacations interfered with the observations. Apart from these gaps the throat swabs were taken once every week.

It will be noted from the charts that the throat cultures often showed a great abundance of meningococci, these organisms occasionally being

present in almost pure culture. Weekly variations in numbers were often very great but, except when the swab obviously had been allowed to touch some part other than the posterior nasopharynx, cultures taken on following days showed numbers of meningococci which corresponded within the somewhat wide margins of error inherent in the method. The percentages given in the protocols and charts are of necessity only approximate. An attempt was made to minimize error by having all of the work done by a single individual and the variations, though not of the exact magnitude shown, are sufficiently accurate for diagrammatic purposes. Often only three, two or even one colony was found on a plate after the most careful search.

Most important, perhaps, in view of the past experience, is the fact that an individual can continue to be a carrier of the organisms and, as far as one is able to ascertain by cultural characteristics and agglutination reactions, can continue to be a carrier of the same strain for long periods, and this despite the fact that careful and repeated swabbings of the nasopharynx reveal no meningococci on the culture plates for periods up to $4\frac{1}{2}$ months. Since this is the case, it is clearly of little use to lay claim to "cures" or to make statements of normal duration of the carrier condition based on negative results obtained in three consecutive weekly swabbings. What happens to the meningococci is uncertain. It may be that they can survive in the depths of the lymphoid tissue crypts, but the present observations offer evidence that a carrier can show apparent cure, as shown by negative cultures, over a period of months and yet at the end of that time become positive again and show a strain apparently identical with that previously isolated.

There are certain interesting features about the typing of the twelve strains isolated from these ten carriers. Four of the strains, those from Nos. 3, 4, 12 and 22, were typical Type II strains differing in no particular from Type II strains isolated from the spinal fluid of cases of meningitis. There were no Type I-III strains. All of the other nine were atypical in agglutination and, at least when freshly isolated, showed no agglutination in sera prepared with freshly isolated Type I and II strains according to the technique outlined in a previous paper (32).

There was available one serum prepared against a nasopharyngeal

strain which had failed to agglutinate in Type I or II serum.[5] The serum prepared against this strain was found to have the property of agglutinating typical Type II strains although the strain itself failed to agglutinate in Type II serum. The strain was next tested for its power to absorb agglutinins and was found to remove them from Type II but not from Type I serum. The strain was called an atypical Type II (designated II*) and the serum prepared against it was called atypical Type II* serum. When the atypical nasopharyngeal strains were tested with this serum, two were found which agglutinated; namely, those from Individuals 4 and 23. These strains did not agglutinate in Type II serum. It is of interest that two strains recently isolated from sporadic cases of frank, and in one instance fatal, meningitis, occurring in children, have proved to be atypical Type II*, identical in every way with those isolated from the apparently normal nasopharynx.[6] It is clear from this that these atypical nasopharyngeal strains are capable of producing the characteristic picture of cerebrospinal fever.

Of the six strains remaining unagglutinated, three were used for the production of antiserum; namely, Strain 438 from No. 17, and Strains 456 and 444 from Individual 8. The serum prepared from each of these strains did not agglutinate any other of the three, at least when the strains were freshly isolated. They were designated Types V, VI and VII respectively. It was found that Type V antiserum would agglutinate Type I-III strains slightly and that Type V strains removed agglutinins from Type I-III serum. Similarly, Types VI and VII showed these relationships with Type II. One other strain, from Individual 13, was shown by agglutination with these new sera to be a Type V and two others, from Individuals 15 and 19, were Type VII.

Table I shows the strains belonging to the different types and the individuals from whom they were isolated. As a result of the relationships pointed out above, Type V is believed to belong to Group I, which includes Types I and III; and Types II*, VI and VII belong to Group II, which includes Type II and probably Type IV.

[5] This strain, No. 441, was isolated from a patient in the Cardiac Service and was obtained through the courtesy of Dr. Thomas Francis, Jr.

[6] Both of these strains came from the New Haven Hospital through the courtesy of Dr. James D. Trask.

After these nasopharyngeal strains had been maintained for months on artificial media it was found that those belonging to Type V showed some agglutination in typical Type I-III serum while Types II*, VI and VII agglutinated in Type II serum, thus confirming the conclusions already arrived at as to the relationships of these strains. It is interesting to note that Scott (6), working on non-contact carrier strains during an epidemic period, isolated strains which were similar to those described here in that they differed from typical Type I-III or II strains but showed some relationships with them.

It will be seen that ten of these carrier strains belong to Group II while only two belong to Group I. There are moreover four typical Type II strains and no Type I-III. It is now fairly well agreed in this country and in Europe that Type I-III is prevalent during the

TABLE I

Group	Type	Strain	Carrier
I	V	427, 438	13, 17
II	II	475, 457, 482, 15	3, 4, 12, 22
	II*	468, 18	4, 23
	VI	456	8
	VII	444, 422, 443	8, 15, 19

epidemics which have been seen in the past decade, while Type II accounts for a large number of the sporadic cases and undergoes a relative increase in prevalence during interepidemic periods.

An attempt was made to keep a complete record of all colds, sore throats or other infections of the upper respiratory tract suffered by members of the group during the period of observation. In the case of the carriers, these are indicated in the charts. The results were too scanty to allow of definite conclusions but certain facts can be pointed out. Coryza which was not accompanied by an overwhelming outgrowth of one of the throat pathogens did not appear to change the numbers of meningococci in the nasopharynx, as judged by numbers on the throat plate. On the other hand, pharyngitis and any infection accompanied by the outgrowth of one of the pathogens, as for example *H. influenzae* or pneumococcus, in large numbers

did cause the numbers of meningococci to decrease and even produced an apparent cure of the condition in one instance. This is of interest in view of the work of Gordon (32) and Colebrook (33) who showed that certain organisms and their products, more particularly the pneumococcus, were definitely antagonistic to the meningococcus and inhibited its growth markedly.

The Foundling Hospital Group

A group of 25 young girls and female infants, from 14 years to 6 months in age and of both white and colored races, were swabbed weekly. These children were all strictly isolated in a separate ward on account of gonococcal vaginitis and remained in this ward until pronounced free from the infection. The length of stay of members of the selected group in the ward varied from 3 to 28 weeks from the time of commencement of the nasopharyngeal investigation. The technique followed was that outlined above. Among the 25 children two were found to harbor meningococci at some time.

Individual A.—Colored infant, 14 months, was found to be positive when first examined on Sept. 8, 1932. Meningococcus colonies of Type I-III were found in 10 per cent of the throat plate. The following week they formed 5 per cent, the 3rd week 40 per cent and the 4th week 20 per cent. On Oct. 4, the 5th week the swab showed no meningococci and the child remained free up to the end of the 10th week when, the vaginitis being cured, she was discharged. In the case of this child it is of course impossible to say how long before the investigation was started the infection began. Moreover, it is not possible to say that the carrier state had spontaneously cleared up since the child was lost sight of at the end of the 10th week after only five successive negative weekly swabs which, it is believed, do not constitute sufficient evidence of a cure.

Individual B.—6 year old white girl. She also carried a Type I-III meningococcus. Since the carrier state began Sept. 27, while Individual A was still positive with the same type, it is possible that this is an example of contact infection. B was negative for the first 3 weeks from Sept. 6 and then on Sept. 27 showed meningococcus colonies as 30 per cent of her throat plate. The next week the organisms were present in 10 per cent but on Oct. 11 she became negative and remained so for 22 weeks until Mar. 14, 1933, when, the vaginitis being cured, she was released from isolation.

The numbers of individuals investigated in these first two groups are strictly comparable but it must be emphasized that the duration of the investigation varied considerably. Thus the average length

of observation in the adult group was 15½ months as compared with 14½ weeks in the children. Nevertheless, the difference in carrier rate between the two groups—41.6 to 8.0 per cent—is striking. Two factors at least would seem to have a bearing on this finding. The first concerns technique. It has been found quite impossible to obtain such satisfactory swabs from children as can be obtained from the majority of adults. The swabs are apt to be contaminated with saliva in spite of all precautions and there is no doubt that the salivary cocci and their products which are not normally present in the nasopharynx do inhibit the growth of meningococci even up to total extinction. It may be that certain transient carriers amongst the children were missed on this account, but probably no chronic carriers escaped notice. The second factor is that of isolation. These children were isolated fairly well from contact with the outside world and thus the possibility of introduction of a fresh strain from the outside was greatly reduced. Carriers would arise probably only from strains already in the group, as indeed may have happened with Individual B. It is striking that there were no Group II strains.

The Forestry Group

569 young men between 18 and 25 years of age, picked from all sections of the community for the Civilian Conservation Corps, were investigated while in concentration at Fort Slocum during the spring. It was only possible to examine each individual a few times. Thus, 83 were swabbed on 3 successive weeks, 291 on 2 successive weeks and 195 only once. Twelve carriers were found and of these one was swabbed three times (positive once), nine were swabbed twice (two being positive both times and seven only once) and two were swabbed only once.

Eleven out of the twelve strains were agglutinated, one strain being inagglutinable when first isolated and then being lost in subculture before the agglutination test could be repeated. Of the eleven agglutinated strains only one, a typical Type I-III, belonged to Group I. The other ten, belonging to Group II were divided as follows: Type II, two strains; atypical Type II*, one strain; and Type VII, seven strains. This percentage of carriers, 2.1 per cent, is a low normal figure and is about what one would expect from such a group examined under

such circumstances. It corresponds to the carrier figures as usually given. It is believed, however, that it is appreciably lower than the real carrier rate among this group of young men, a rate which could be determined only by careful weekly examinations made under conditions as favorable as those that could be applied to the Rockefeller Institute group.

The proportion of Group II strains is again noticeably high and corresponds very closely to what was observed in the Institute group and to the figures given by Scott and others. It would seem that the members of Group II, including strains apparently identical with Type II strains obtained from the spinal fluid of meningitis patients, are those which are to be expected most commonly as apparently harmless saprophytes in the throats of normal individuals.

SUMMARY

Of the three studies which have been reported in this paper, the most thorough and therefore the most instructive was that made upon the Rockefeller Institute group of 24 individuals. The ten carriers discovered in this group were found to fall into three categories; namely, chronic, intermittent and transient carriers. It is, perhaps, a matter for surprise, in view of the weight of evidence in the literature, that half of the carriers should appear in the chronic group, being constantly affected for periods over 2 years and continuing to carry throughout this period what was, to all tests, the same strain of microorganism. It has been shown that no claim of relief from the carrier condition can be based on three consecutive negative swabs at weekly intervals since apparent spontaneous "cures," as evidenced by negative swabs, may last for $4\frac{1}{2}$ months and finally be terminated by the reappearance of the same strain as that carried before.

The effect of coryza and pharyngitis on the persistence and degree of the meningococcal infection has been studied and, while the results are scanty, indications have been found that coryza, unassociated with any increase in numbers of the nasopharyngeal pathogens or streptococci, causes no change in the number of meningococci present in the throat. On the other hand, a streptococcal pharyngitis or any infection in which other throat pathogens increase greatly in number

is usually associated with a marked diminution or actual disappearance, whether temporary or permanent, of the meningococci from the nasopharynx. This is in accordance with the work of Colebrook and Gordon.

Of the 26 carrier strains which were isolated in these three groups of individuals, only eight could be identified with Gordon's four types which are isolated from the majority of cases of meningitis. It is considered as certain, however, that the other 18 strains are to be regarded as true meningococci. Not only do they show the same cultural characteristics and fermentative reactions as the typical strains, but serological tests, especially that of absorption, have revealed that they are allied to the two main types, I-III and II, and can be regarded as belonging to the broad serological Groups I and II which include these typical Gordon types. Moreover, atypical Type II* strains, identical with those isolated from the nasopharynx of carriers, have recently been found to be the cause of two cases of frank cerebrospinal fever. Only five of the 26 strains belong to Group I while the other 21 are members of Group II. This is interesting in view of the work of Scott who found that Group II strains predominate in carriers during interepidemic periods like the present. In periods of epidemics the carrier strains from both contacts and non-contacts in the epidemic zone are more often of Group I and even more constantly tend to be of the typical Gordon types rather than atypical forms.

As has been pointed out in an earlier paper (35), the viability of these carrier strains when planted in defibrinated rabbit blood is low as compared to the typical and freshly isolated meningitis strains. The exact significance of this fact is not known. It has not been possible up to the present to do comparative virulence tests between spinal fluid and nasopharyngeal strains owing to the absence of a sufficiently susceptible animal.

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SELECTION WITH THE MAGNET AND CULTIVATION OF RETICULO-ENDOTHELIAL CELLS (KUPFFER CELLS)

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The existence on the walls of the small blood vessels within certain organs of multitudes of cells endowed with remarkable phagocytic ability has long been recognized. To the older students of body processes these cells appeared to have a sufficient task in the removal and disposal of bacteria, effete corpuscles, and other particulate matter. Recent observations and the growing realization that cells of a single sort may do several kinds of work have led to a renewed scrutiny of the capabilities of the fixed phagocytes. They are now supposed by many investigators to constitute a distinct physiological system, the reticulo-endothelial system, so-called; and functions in great variety have been ascribed to them.

The reticulo-endothelial cells may very well carry out important tasks besides scavenging. Much evidence points to this. But it has been obtained under complicated conditions and hence has remained inconclusive. The innumerable attempts to produce a functional blockade of the cells by inducing them to gorge themselves with particulate matter have inevitably disturbed the organism in other ways, as have also the tests wherein the cells have been led to take up particles which poison and kill them. The need to procure and maintain living reticulo-endothelial cells for study *in vitro* is an obvious one. By the methods here to be described, this has been done for the reticulo-endothelium of the liver (the Kupffer cells).

Flushing the Cells from the Liver

Forcible washing out of the normal dog or rabbit liver through the portal vein, or backwards through the hepatic vein, even when com-

bined with intermittent distention under pressure and kneading, causes few or no Kupffer cells to be dislodged, and the yield is equally poor whether the fluid be serum, or Tyrode containing $\frac{1}{8}$ per cent of gelatine, or ordinary Tyrode, at room or body temperature. Into chilled fluid the Kupffer cells do not come away at all, and attempts to shock them off the wall of the liver sinuses by adding a trace of formalin to the wash fluid, to digest them off with trypsin, or to anesthetize them into letting go their hold by means of perfusion with a fluid containing ether, have alike proved unavailing.

Von Kupffer (1) noted that after the cells had taken up particulate matter some of them came away into the venous blood; and later students of vital staining have described "showers" of dye-laden Kupffer cells in blood procured from the right heart. A major difficulty in the attempts to "block" the cells with India ink and other materials has been their rapid proliferation with result that great numbers of new, unblocked ones soon come into being. These observations have led us to inject animals several times intravenously with particulate matter, washing out the liver forcibly a few days later when one might suppose that the cells could be dislodged from the capillary wall.

The initial experiments were carried out with India ink. Rabbits were injected intravenously at intervals of a few days with 5 to 10 cc. of a half strength preparation of Higgins American Drawing Ink, a non-waterproof suspension, which had been dialyzed in the cold for 10 days against several changes of sterile Locke's solution and filtered through paper. Three or four injections of 20 cc. were made into dogs, and of 5 to 20 cc. into rabbits.

At various periods after the last injection the animals were anesthetized, the inferior cava was ligated above the kidney, and the liver was perfused with warm Tyrode solution through the portal vein, with collection of the washings from the superior cava. Flow was begun at a pressure of 10 cm. Tyrode, and when the washings no longer contained blood the pressure was raised to 30 cm. and finally to 60 cm., with intermittent obstruction to the outflow, and kneading of the distended liver as it emptied itself after the obstruction was removed. The fluid first collected on raising the pressure was a cloudy, dark gray, owing to the presence of myriads of ink-laden cells. These phagocytic cells were of several sorts, many being polymorphonuclear leukocytes. When the flushing out was done on the day after the last ink injection, as much as $\frac{1}{2}$ cc. of them could be obtained from the liver of a 2000 gm. rabbit, though the yield was usually less. If on the other hand washing was done after an interval of a week or more the yield of phagocytes was very small.

The tests showed that when the conditions were rightly chosen great numbers of phagocytes could be obtained. Not a few were present with blood in the first perfusate at low pressure. The morphology of the cells and their significance is considered in an accompanying paper. Many were enormous as compared with the accompanying leukocytes, and there was no difficulty in identifying these as Kupffer cells. On coming in contact with a glass surface, in the warm box, they put forth a broad membrane and then were often as much as 100 μ across (Fig. 1).

The ink-containing cells, though sedimenting rapidly, could not be separated entirely from the associated elements by differential centrifugation. It was found, though, that if iron had been taken up instead of ink, selection could be accomplished with the magnet.

For the first attempts minute spherical iron particles (alcoholized iron, Merck), selected from the commercial preparation by differential sedimentation, were injected in suspension in a solution containing 7 per cent gum acacia and 0.9 per cent NaCl. They were taken up by Kupffer cells, some of which were found in the liver washings; but the heavy material so weighted the cells that they were incapable of movement. Resort was now had to the gamma ferric oxide of Baudisch and Welo (2), a relatively light substance of strong magnetic properties.[1] The pure material was suspended to the amount of 4 per cent in 14 per cent gum acacia made up in distilled water, and ground in a colloid mill (No. 1 High Speed Laboratory Mill of the U. S. Colloid Mills Corporation)[2] until the particles were 1 μ or less in diameter. Distilled water was then added to make a 2 per cent suspension of the iron oxide in 7 per cent gum acacia, and the suspension was autoclaved in test tubes under washed paraffin oil to rule out oxidative changes. The larger particles tended to sediment during storage in the ice box, but were readily resuspended for injection. We have gained the impression that preparations made within 2 or 3 weeks of use yield the best results in terms of free Kupffer cells. The iron oxide, as seen within these cells, consists of roughly spherical, amber-brown particles. They persist unchanged within the cells. The liver of a rabbit killed 19 months after injection yielded phagocytes containing the characteristic, irregular, brown particles, and these obeyed the magnet and gave a positive potassium thiocyanate test.

[1] We would stress out great indebtedness to Dr. Oskar Baudisch for his repeated generous gifts of material and for his interest in the work.

[2] The material was ground for us through the kind interest of Mr. M. P. Hofmann of the Corporation.

Method to Procure Kupffer Cells

On the basis of these orienting observations the following method was developed:—

A rabbit of approximately 2000 gm. is slowly injected by way of an ear vein with 10 cc. of a sterile 2 per cent suspension of gamma ferric oxide in 7 per cent gum acacia, and the injection is repeated twice at intervals of a day. Albinos should not be used since their cannulated portal and hepatic veins sometimes rupture under the perfusion pressure. The washing out is done 3 days after the last injection. The animal should be fasted in the interval to reduce the size of the liver and render it less friable.

Under ether the shaved abdomen is opened widely in the mid-line, the right renal and adrenal veins are ligated together, and the inferior vena cava is freed from about 2 cm. below the entrance of the right renal vein to where the liver is attached to it. Two threads are placed at either end of the freed segment but left untied. With due care not to interrupt the blood flow, about 3 cm. of the portal vein is freed in turn, just below where it forks before entering the liver masses, the tributaries to the segment are tied, and two ligatures are carried under the portal but left loose. A stout thread is tied down on the gastrohepatic omentum to close off the hepatic artery.

The lower of the ligatures around the inferior cava is now tied, the anterior one is lifted to obstruct the vessel temporarily, preventing a back flow of blood, a slit is made just above the posterior ligature, and the cannula connecting with the perfusion apparatus is inserted toward the diaphragm. The obstructing ligature is relaxed so that the cannula can pass it, and is then tied down. At once fluid is allowed to enter at 10 cm. water pressure, and then the chest is widely opened by cutting transversely across the sternum and between the ribs in the 5th interspace; a thread is thrown about the superior cava about 2 cm. below the heart; a cut made in the vessel to allow the segment of cava back of the liver to be flushed free of blood by the wash fluid; and the ligature is tied below the cut, and the heart and the portal vein are opened wide with scissors. In this way circulation through the liver is maintained until the moment comes for the backward flushing out with the wash fluid. All operating is done as rapidly as possible, but with due precautions for asepsis. If there is a delay in the washing, many of the Kupffer cells will have leukocytes clumped upon them.

The portal vein can now be cannulated at leisure, the upper ligature upon it serving for this purpose and the lower to block off blood flow from the intestines. The wash fluid is Tyrode solution[3] sterilized by Berkefeld filtration and warmed to approximately 40°. With this as good a yield of Kupffer cells is obtained as with homologous serum. Tyrode tends to become more alkaline on standing.

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[3] $\frac{1}{2}$ per cent gelatine Tyrode was used in the earlier separations (3); but experience has shown the gelatine to be unnecessary.

For this reason phenol red should be added to it before filtration, in the proportion of 4 cc. of a watery 0.3 per cent solution for every 4 litres, and CO_2 bubbled through until the color becomes orange-pink, indicating a reaction of about pH 7.3. The warmed wash fluid flows to the liver through a syphon from a 6 litre Florence flask. The washings are collected from the portal vein through a rubber tube with a glass connection projecting through a stopper into an inverted funnel with cut-off stem. The flange provided by the funnel serves to protect the fluid from contamination during collection.

At 10 cm. pressure the fluid flows but slowly through the liver, and while it is flushing out the blood the suspensory ligaments are cut through and the gall bladder is slit and mopped out. All is now ready for the flushing away of the Kupffer cells.

To aid in the removal of the last blood, the liver lobes are gently lifted from time to time, and when the wash fluid comes away practically clear collection is begun, at first of samples into 10 cc. cylinders in which 0.1 cc. of a sterilized 1 in 1000 heparin solution has just been placed. Then the pressure is raised to 30 cm. and the further washings are received in a pyrex vessel of about 300 cc. capacity, ("taper flask") shaped like a narrowed separation funnel (Text-fig. 1), with straight sides to minimize the accumulation of sedimented cells. The flask contains 1 cc. of 1 in 1000 heparin solution in saline. Just before the pressure is raised the inflow is stopped and the right hand is thrust palm up under the liver so that the first and second fingers lie on the left side of the cava and the fourth and fifth on the right side. The fluid is then turned on and, as it flows through, the liver is gently kneaded. Soon the outlet tube is clamped, the liver is allowed to swell moderately, the outlet is opened again, and massage is done gently but firmly with the left hand to aid in expelling the fluid. This alternate swelling and massage of the liver as it empties is repeated several times with short waits between, during which as the findings show, the Kupffer cells tend to loosen. The entire procedure from the start of the operation requires about 20 minutes. A final sample is taken from which, with the earlier, one can learn whether iron-containing cells are present in quantity.

The same procedures are used with dogs but they are given much more iron, 30 cc. each day for 3 days, with washing out on the 3rd day after the last injection. The dog liver yields far more phagocytes than the rabbit and it can be massaged with less danger of rupture. Furthermore the Kupffer cells survive longer in Tyrode.
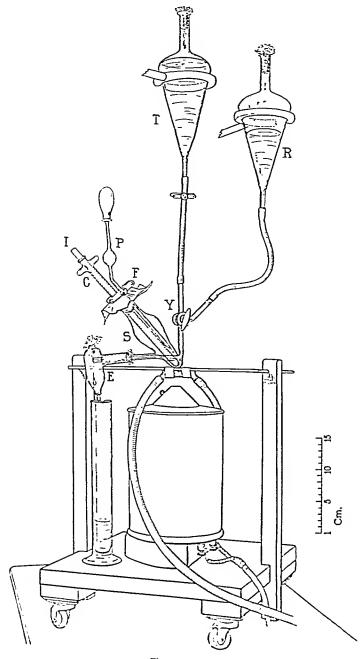
Selection of the Cells with the Magnet

It was essential for the ultimate objects of the work that the cell suspension should be handled under conditions excluding infection, that the phagocytes containing iron should be drawn upward by the magnet,—to avoid an admixture with sedimenting elements of other

sorts,—and that after collection they be held in place while Tyrode flowing past removes all traces of blood. A further essential not foreseen as such was to collect the cells on a surface from which they could be readily dislodged. For when they had been attracted to a glass surface a strong stream was required to bring them away and a considerable proportion were killed during the process. After many modifications, a satisfactory apparatus has been developed (Fig. 2 and Text-fig. 1).

A large unipolar electric magnet (Giant Eye Magnet of the General Electric X-Ray Corporation) is fixed vertically beneath a glass chamber into which the cell suspension is run. It flows in a thin layer under and around a long glass tube having on the outside a collodion membrane and inside a rod of soft iron. When the current is passed through the electric magnet, this rod becomes magnetized secondarily and if the distance between the two has been properly adjusted, its pull upwards is considerably greater than the downward one, sufficing to attract and to hold practically all of the iron-containing cells present in the layer of slowly moving fluid round about. They collect on the collodion membrane and are washed *in situ* with Tyrode solution, run through with enough rapidity to flush out and remove all sedimented cells from the separation chamber. The tube and iron core are then taken out together, the cells remaining attached because the core has now some magnetism of its own; the end of the tube is dipped in rabbit serum; the iron core is withdrawn; and the cells are shaken off into the fluid. They can then be cultured.

Details of the apparatus are given in Fig. 2 and Text-fig. 1. The neck of the taper flask (*T*) connects with a rubber tube which is closed off with a screw-clamp until it is slipped over the end of the tube which leads to the separation apparatus. The latter (*S*) is set up in a room with air freed of bacteria by spraying and air filtration. The mouth of the taper flask is closed with a tight-fitting cotton plug, so that it can be inverted at will to keep the cells in suspension, and it rests in a ring stand with an opening at one side of the ring to facilitate removal. The *Y* tube with which it connects vertically,—to give less chance for sedimentation,— has a three-way stop-cock at the fork. The slanting limb of the *Y* connects with a second flask containing Tyrode solution. Flow into the separation chamber is regulated with the stop-cock, and its rapidity is gauged by the outflow tube which is protected by a glass shield (*E*).
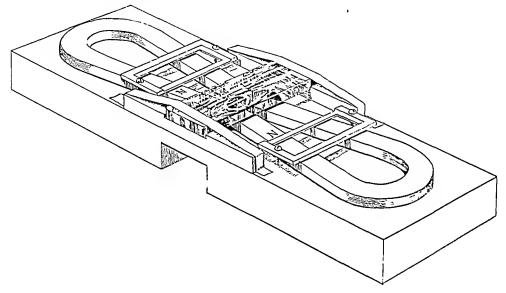
The separation chamber is shaped like an elongated pear. It has a broad mouth

TEXT-FIG 1

closed with cotton covered with Chinese silk to prevent fibres from falling into the fluid.   Two tubes pass through the stopper, the glass "core tube" through its center, and, through its uppermost side, a narrow pipette (*P*) provided with a bulb and a large rubber nipple.   The pipette is used to draw off all possible fluid from about the core tube, prior to removal of the latter from the apparatus.   Until that time the point of the pipette is not pushed down into the fluid.

The core tube (*C*) has a cross-piece near the top for ease in handling.   Around its lower portion is a collodion sheath not shown in the drawing, made by dipping this portion into a concentrated sucrose solution and, when this has dried, dipping a little more deeply into a collodion solution.   Before the latter has completely

TEXT-FIG. 2

dried the tube is immersed in several changes of water to dissolve out the sugar. The result is a sheath attached at its top to the tube, but not elsewhere, and hugging the glass closely after sterilization.   When the separation chamber has been assembled it is autoclaved, with a little water in it to prevent the collodion from becoming brittle.   Before the liver washings are run in, the core tube is adjusted and held with its lower end about 2 mm. above the bottom of the separation chamber, by means of the rubber-covered screw-clamp (*F*).   Two pointed glass studs (not shown), projecting into the separation chamber a little way up from the bottom, prevent it from being pulled against the lower side of the apparatus when magnetization is begun.   A soft iron rod (*I*), which fits it snugly and has a rounded end, is then inserted.

The separation chamber rests on a heavy glass plate held in cleats between two uprights.   Under it is fixed a glass chamber in which water circulates, to prevent heating when the magnet gets warm.

After the taper flask has been connected with the apparatus the water in the separation chamber is replaced with Tyrode's solution by turning the three-way stop-cock, and then the magnet is brought into play and the cell suspension is slowly run in. It flows under and around the bottom of the core tube. A rate of about 1.5 cc. per minute has proved best for collection. When iron-containing cells are plentiful a brown skim, soon thickening to a fur, collects on the core tube. If the magnet is allowed to get very hot its pull falls off greatly. To prevent this we have regulated the current by means of a rheostat in such wise that the magnet exerts a pull of 1.13 kg. at a distance of 4.75 cm. on a cylinder of cold rolled steel shafting 5.06 cm. in diameter and 18.25 cm. long, held vertically over its pole. With this amount of current our magnet does not overheat and the strength of the pull is maintained.

When the collection of the cells is finished,—a matter of 2 to 3 hours, depending on the amount of material,—the separation chamber is flushed out with a brisk current of Tyrode solution from the reservoir (R), thus washing the cell material while still in place. Then by means of the pipette as much fluid as possible is drawn off. This is important since the higher the meniscus the greater is the likelihood that some of the fur of iron-containing cells will be lost when the core tube is withdrawn. It is now lifted out, stopper, core, pipette, and all, without turning off the magnet; and its cell-coated end is at once submerged in sterile rabbit serum. Transfer without loss is possible because secondary magnetization of the iron rod holds the cells in place after removal from the neighborhood of the large magnet. The rod is now slipped out of the collecting tube, and the bulk of the cell material falls away immediately into the serum. Most of that which still adheres to the collodion can be dislodged by cutting this up and gently agitating and pipetting the fragments. Several tenths of a cubic centimeter of Kupffer cells can be obtained from the liver of a 2000 gm. rabbit.

Cultivation of the Cells in Vitro

Attempts were first made to grow the cells in a plasma clot according to the usual technic. They were dislodged from the collecting tube into Tyrode solution, and 3 parts of the resulting cell suspension were mixed with one of rabbit plasma and plated in amounts of 2 cc. in Carrel dishes. In cultures thus made the cells sedimented to the glass before clotting occurred, and their morphology could be readily studied. Few put out membranes during the succeeding days of incubation, though many survived as large spherical, or slug-shaped, entities. When first plated they were frequently in aggregates, as collected by the magnet, but in the course of 48 hours at 37°C. these broke up, the cells slowly moving apart (Fig. 3).

Some division of the cells took place (Fig. 4), yet the fact soon be-

came plain that in even the thinnest clot they could not be propagated in quantity nor could reliable tests of their functions be carried out. Consequently a nearer approximation to natural conditions was sought.

Within the liver the Kupffer cells live on the walls of small channels with a current of fluid flowing past them; and histologically they appear to be fixed on the walls by a broad membrane. They put out such a membrane on coming in contact with a flat glass surface when they are first procured. Normally it is extended on the inner side of the curve of the capillary wall; but there seemed some likelihood that extension would also occur on the outer side of a curve or in other words that the cells would fix themselves and live on the outside of fibres bathed in fluid, in much the same way as on the interior of small vessels. This has proved to be the case. When a suspension of the cells in serum, as first procured, is poured upon a layer of washed sterile lens paper in a Carrel dish, they fall through the interstices of the paper to the bottom of the dish. Here some of them flatten out and remain. The majority, however, swarm up the fibres within the first 48 hours of incubation (Fig. 6) and scattering, fix themselves here and there. Their arrangement may be likened to that which would exist if the liver capillaries were turned inside out. Immense numbers, distributed on the fibres, can be kept in flourishing condition if the serum is replaced each day (Fig. 5). The following technic has been developed:—

Leitz lens paper, non-ribbed, is washed in several changes of 5 per cent hydrochloric acid, rinsed in distilled water until acid-free, dried, and cut into discs that will cover the bottom of Carrel dishes 3 cm. in diameter. Two discs are introduced into each dish, spread flat with a glass spatula and autoclaved. The Kupffer cells selected with the magnet are shaken or pipetted away into serum,—in amount regulated by the number of cultures desired,—and 2 cc. of the suspension is distributed to each dish. The latter are closed with rubber stoppers and incubated 24 to 48 hours, that is to say until the cells have put out membranes and fixed themselves on fibres or glass. The serum is changed daily thereafter by tipping the dish and drawing off as much as possible, usually about 1.8 cc., or nine-tenths of the whole. Enough remains between the fibres for the cells to remain undisturbed. They have been successfully maintained for as long as 10 days, during which period an active multiplication of them took place. We have not tried to keep them longer since it seemed best to study their functions as soon as possible.

When the cells were propagated in serum diluted with Tyrode they showed many small granules of fat after the first few days, and tended to round up and fall from the lens paper. Successful cultures in undiluted serum showed practically no fat, and the cells remained more or less flattened.

Effect of the Magnet as Such

Under the influence of the magnet most of the cells collect along the lines of force and the intracellular iron particles undergo a regrouping, with result that cells and iron, on suspension in serum, may appear like cherries skewered on a stick, the iron particles of the cells being ranged in a line to form the stick. It was noted that the cells of such configurations seldom survived in culture. To test whether magnetization, as such, kills cells containing ferromagnetic iron oxide, the following experiment was done:—

Two small horseshoe magnets were screwed to a board, with the north poles opposite each other at a few millimeters distance (Text-fig. 2). Under aseptic conditions a few drops of a freshly procured suspension in serum of iron-containing cells were placed within a deep ring of vaseline in the well of a large hollow-ground slide, a cover-slip was sealed on, and the slide was turned over and fixed on raised cleats to either side of the magnet, so that the vaseline ring,—which extended from slide to cover, retaining the fluid,—lay between the magnet poles, with the cover-glass just clear of these. A hole through the middle of the wooden block on which the magnets were set permitted study of the cells by transmitted light. Incubation was done as usual. The iron-containing cells promptly fell out of suspension onto the cover-glass. The majority were drawn into the lines of force within the first minutes, and the intracellular iron particles were seen to shift position gradually until they had become arranged in apparently continuous lines (Fig. 7). Not a few cells which happened to have fallen between the lines of force fixed themselves there, and the arrangement of the iron they contained did not indicate any influence by the magnet. These served as controls. In preparations incubated for 4 days without change of the medium they fared no whit better than the ones skewered on the lines of force. Both put out membranes (Figs. 8 and 9), and underwent gradual fatty changes to the same degree. Preparations that were removed from the influence of the magnets after 1 or 2 days, and further incubated, showed a prompt breaking up of the linear fixation of the cells, these moving off in all directions along the glass.

The experiment shows that sufficient magnetization of the intracellular iron to cause alignment of it and a grouping of the cells along the lines of force causes no harm. It is possible, of course, that strong magnetization is lethal in effect. However a sufficient explanation

of the cell death we observed as result of it is to be found in the brusque rearrangement of the intracellular particles that it causes. Chambers and Fell (4) have demonstrated that tearing the cytoplasm of a mammalian cell with a micro-needle suffices to kill it. The Kupffer cells are stabbed from within so to speak. That they are extremely sensitive to trauma is shown by the fact that most of those dislodged by very forcible liver washing are dead when obtained.

Infection as a Complication

The vascular arrangement whereby blood coming from the intestines must pass by the Kupffer cells, and the known special ability of these cells to take up and destroy bacteria, suggest that they are constantly engaged in removing bacteria which have entered the circulation from the gut. Many experiments have been reported which have been taken to support this conception. In the material utilized by us for cultivation there were always Kupffer cells that had died during the manipulations; and *a priori* it seemed all too likely that bacteria recently phagocyted by some of these, but not destroyed prior to their own death, would overgrow the cultures, especially when the bactericidal action of the serum had been lessened by heating it at 56°, as was frequently the case. Such overgrowth did occur on occasion, the bacteria rapidly multiplying throughout the fluid medium. This happened almost regularly when the cells were obtained from the livers of animals that had appeared to be ailing. In the attempt to obtain a larger yield of iron-containing cells than ordinary, some rabbits and dogs were given one or several intraperitoneal injections of a sterile solution of nucleic acid, while others received a suspension of killed cultures of *B. prodigiosus* intravenously. They had fever in consequence and the washing out of the liver was done while it was still present. The yield of Kupffer cells under such conditions was extraordinarily abundant, but the cultures were promptly overrun with bacteria. On the other hand cultures from normal rabbits and dogs remained uninfected when there had been no slips in technic. It was clear that either the bodily derangements caused by the injections so affected the Kupffer cells that they could not cope with incidental bacterial invaders phagocyted by them, with result that these were carried with them into the cultures, or else that blood

invasion by bacteria capable of growing in cultures containing Kupffer cells in serum (whole, or inactivated, or diluted with 3 parts of Tyrode) occurs to but a slight extent if at all normally. The latter alternative seems the more likely, since the Kupffer cells washed out of the animals with fever, instead of appearing to be adversely affected, were notably more active than usual, swarming up the strands of lens paper and scattering upon it after only a few hours of incubation instead of the usual 24 to 48,—within which period, however, they died in consequence of profuse bacterial growth. In rabbits with fever, whether produced by nucleic acid, killed bacteria, or by infection, living and active Kupffer cells could be washed from the liver when there had been no preliminary injection of particulate material. An animal washed out at the height of vaccinial infection gave a notably large yield. As already stated washings from normal animals contain only an occasional Kupffer cell.

These facts indicating that the Kupffer cells are markedly influenced in their activity by the general body state, as further, perhaps, that non-specific disturbances favor the entrance of bacteria into the blood stream, have as yet not been followed up experimentally.

SUMMARY

Methods and apparatus are described wherewith living Kupffer cells can be procured from the liver of the rabbit and the dog for study and cultivation *in vitro*. Almost none of these cells can be dislodged from the normal liver by forcible perfusion; but after they have taken up finely particulate matter (India ink, iron oxide), they come away in great numbers. When they have phagocyted ferromagnetic iron oxide they can be selected with a magnet from amongst the blood elements present in suspension with them; and they are obtainable in quantity by this means. They do poorly when plated in a thin plasma clot, failing to multiply or to assume their characteristic shape; but they flourish when allowed to attach themselves to strands of lens paper bathed in serum that is frequently changed.

Bacterial infection of serum cultures of Kupffer cells from normal rabbits and dogs occurs only as the result of secondary contamination of the materials, whereas it regularly develops in cultures from animals with fever induced by the injection of nucleic acid or of killed *B*.

prodigiosus. Kupffer cells obtained under such conditions are abnormally active, and some can be washed out of the liver of sick animals in the absence of any preliminary phagocytosis of particulate matter. The facts have a bearing both on the conditions conducing to blood invasion and on the response of the Kupffer cells in the emergency.

The characters of the isolated Kupffer cells and the results of tests of their presumptive functions will be described in later papers.

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EXPLANATION OF PLATES

All of the photographs were made from fresh preparations.

PLATE 40

FIG. 1. Slide and cover-slip preparation of the liver washings of a rabbit repeatedly injected with India ink,—to show the relative size of erythrocytes, nonphagocytic white cells, and Kupffer cells with membranes extended on the glass. The photograph was taken slightly out of focus since otherwise the membranes would not be seen. Their edges are crinkled as result of partial retraction induced by the strong light. × 400.

FIG. 2. The separation apparatus ready for use.

PLATE 41
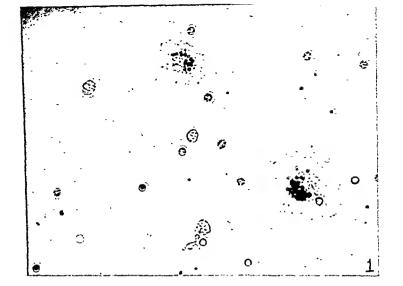
FIG. 3. A clump of iron-containing Kupffer cells from the dog, breaking up as the cells slowly disperse. Photograph taken after 48 hours incubation in a thin plasma clot. × 180.

FIG. 4. Part of the same culture at a higher magnification. Three pairs of cells can be seen, resulting from *in vitro* division. × 360.

FIG. 5. A culture of iron-containing rabbit Kupffer cells on lens paper in serum, after 4 days incubation. × 115.

FIG. 6. Iron-containing, rabbit Kupffer cells climbing a fibre of lens paper in a serum culture. Photograph taken through the bottom of the dish. The cells
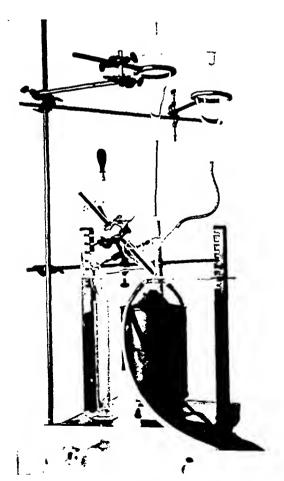
at either end of the fibre lie spread upon the glass.   Their membranes cannot be seen.   × 400.

FIG. 7. Dog Kupffer cells living in the field between two horseshoe magnets. Many of them have been drawn into the lines of force as is indicated by the linear arrangement of the iron they contain.   Impure culture, containing many blood leukocytes; photographed on the 3rd day of incubation.   × 135.
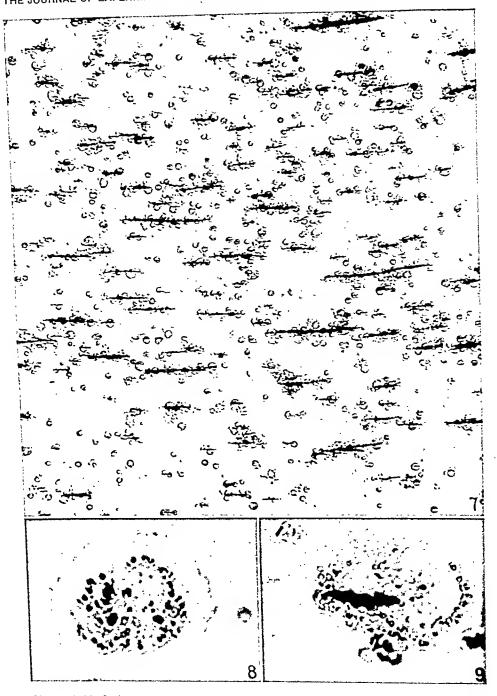
FIGS. 8 and 9.   Two cells of the same preparation, photographed on the previous day.   One lies in a line of force, as shown by the linear arrangement of the intracellular iron, the other outside this influence.   They are in equally good condition.   × 1100.

1

2

PLATE 41

PLATE 42

7

8

9
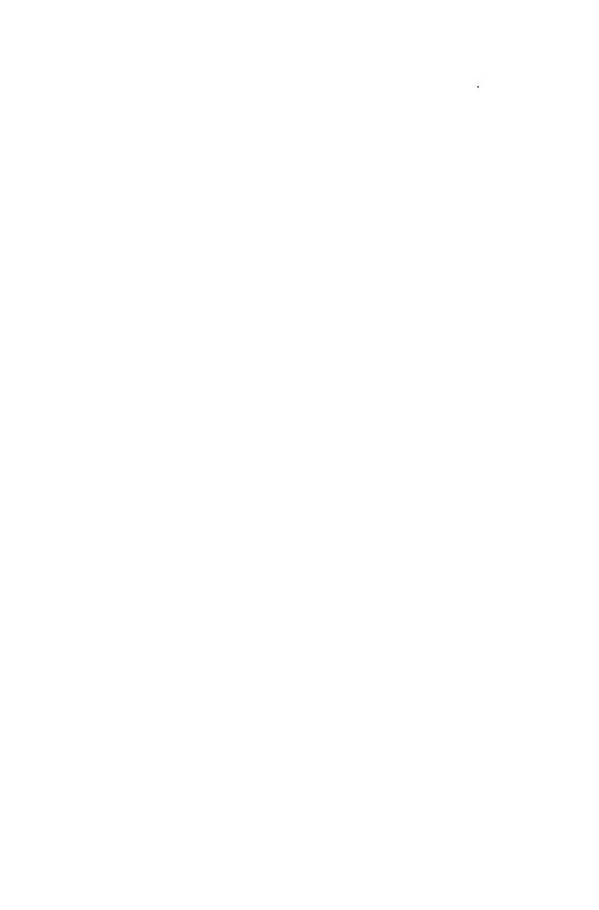
(Rous and Frand: Selection of Kupffer cells with magnet)

THE CHARACTERS OF KUPFFER CELLS LIVING IN VITRO

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(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 43 AND 44

Methods whereby the large phagocytes of the liver sinuses can be procured and propagated outside the body have been described in an accompanying paper (1). The present communication deals with their identification as Kupffer cells, their morphology and general characteristics. Their physiological potentialities under the conditions of life *in vitro* will be dealt with later.

The Kinds of Cells Washed from the Liver

The method of washing out the cells has already been described. The first Tyrode solution put through a normal liver at low pressure (10 cm. Tyrode) removes only the blood. The erythrocytes come away far more readily than the leukocytes. Even long washing, with pressure gradually raised to 105 cm., does not remove all of the latter, sections of the washed liver showing an occasional polymorphonuclear cell in the sinuses and not infrequent lymphocytes and monocytes. It has seemed possible that these leukocytes might have settled out of a slowed blood stream, though the method of cannulation involved no interruption of the hepatic circulation; but they are found even when the washing is done under pressure, through a large trochar thrust into the portal vein while the circulation is intact. The possibility of a settling out of cells is still not excluded, since the blood current may have slowed as a result of the etherization. However this may be, there is no doubt that the washings from the normal liver contain far more leukocytes than can be accounted for by the blood content of the organ at any one moment; and during infections or after the injection of nucleic acid immense numbers of them can be flushed from the hepatic sinuses. The washings from a 6.25 kg. dog with pneumonia

yielded several cubic centimeters on centrifugation. The findings support the old assumption that the leukocytes may dally on occasion in some of the abdominal viscera, thus affecting counts made on the peripheral blood; and they corroborate the histological observations of numerous authors who have reported that lymphocytes and monocytes are normally present in considerable numbers in the liver sinuses. No evidence has been found, however, that the organ serves as a graveyard for white cells. Those washed out have regularly proved to be in excellent condition, when studied in the warm box.

Flushing the liver at 30 cm. to 105 cm. pressure straightway after the blood has been removed brings out not only leukocytes in progressively lessening numbers but a multitude of globular, colorless, slightly refractile bodies of various sizes. These have many of the characters of red cells, being laked by water, bile, and other hemolysins, notably serum hemolysins when complement is present. They show a reticulum with cresyl blue, are agglutinated by specific agglutinins, and leave shadows when laked. Yet, as a previous paper has shown (2), they are products of the liver parenchyma extruded during the first minutes of perfusion.

In most cases these bodies and a greater or less number of leukocytes and platelets are the only yield of the late washings from the normal liver. Very occasionally though one encounters a spherical element 30 to 40 mm. in diameter which flattens on contact with a glass surface and puts out a great circular membrane. The washings from animals injected with India ink or ferromagnetic iron oxide several days previously yield such cells in enormous numbers, each now containing the particulate matter (Fig. 1). They differ greatly from the blood cells, and it is plain that they are inhabitants of the liver sinuses.

Numerous experiments were done to find the conditions which would give the most abundant yield of the large cells. These have shown that not only the number but the kind of phagocytes varies with the interval elapsing between the last injection of particles and the washing out. When only 1 day has elapsed polymorphonuclear leukocytes containing several small particles are fairly frequent, and there are considerable numbers of typical monocytes that have phagocyted particles, in addition to the peculiar cells above mentioned. The sinus cells, as we may call these latter, are much bigger

than the blood elements, nearly always contain numerous particles, and frequently are so crammed with them as to be greatly enlarged. In washings procured 3 to 5 days after the last injection the polymorphonuclear phagocytes have disappeared and there are fewer of the monocytic type. This is the period at which the large sinus cells are most abundant. Washings obtained 2 to 3 weeks after the last injection of particles contain relatively few sinus cells, but phagocytic monocytes are more infrequent still, their proportion as compared with that of sinus cells being much reduced. Sections of the liver at this time show most of the phagocytes gathered into large clumps here and there, with fusion and giant cell formation in pockets in the liver parenchyma. On backward washing through the hepatic vein to the portal an occasional giant cell comes away; but most are so large and so well ensconced as not to be dislodged. Sometimes a giant cell comes away so large that it can readily be seen with the unaided eye. One having a diameter of 540 μ was washed out of the liver of a dog repeatedly injected with iron oxide; and cells measuring 200 to 300 μ are procured with relative frequency (Fig. 2). That they are living is shown by their prompt segregation of neutral red into the numerous vacuoles; and they survive in cultures.

Even forcible washing and massage bring out only a small proportion of the phagocytic sinus cells from the liver,—which remains black or rusty brown, according as it contains ink or iron oxide. Inspection under the microscope of scrapings of the cut surface shows numerous large phagocytes scattered within the tissue fragments, many of them with processes that extend into crannies between the parenchymal cells. In stained sections the sinuses appear swept clean save for these securely fastened phagocytes and the endothelium proper. The loosely attached Kupffer cells have been swept out practically *en masse*, a fact the more evident when the section is compared with one from a piece of the same liver snared off before washing was begun. Search shows, however, both monocytes and lymphocytes in and about the sinuses. A little ink or iron is present here and there within the sinus endothelium.

Characters of the Dislodged Kupffer Cells

The sinus cells have been studied in deep preparations made by putting a few drops of the washings within a ring of vaseline on a slide coated with dry neutral red in the usual way (3). The cover-glass is put on slantingly to expel the air from within the ring and the preparation seals itself with the vaseline. It is then turned upside down in a warm box surrounding the microscope and, supported at its ends, is left undisturbed for about 20 minutes. During this period the phagocytes settle to the cover-slip where the greater proportion become attached. When the slide is turned right side up most of the other elements and the debris fall away, and the adherent cells can be studied through an oil immersion lens, without any complicating pressure upon them. That they are in the main Kupffer cells is certain not only from their morphology but from the fact that these have been largely swept from the liver. At the period when they are most abundant in the washings they are so easily dislodged as to come away in considerable number with the blood of the first washing. Those then obtained are mostly smaller than the ones got later at higher pressures, but they are practically all alive, segregating neutral red promptly. The living Kupffer cells are spherical or ovoid when in suspension, whereas dead ones may be shaped like blunt casts or cigars, or be star-shaped, or flattened, or show twig-like projections, sometimes combined with forking so that they resemble elk horns. Frequently they are like fused spherules of several different sizes, the projecting portions of each spherule consisting of clear cytoplasm. In all these dead cells the outline of the nucleus is sharp cut; and they fail to segregate neutral red though the nucleus stains readily with trypan blue. Some have ragged protoplasmic processes as if they had been forcibly torn from the capillary wall; and that this actually happens, with death in consequence before the cell has had time to change shape, has been shown by counts of the living and dead elements in specimens of the successive washings at higher and higher pressures. In the final washings at 105 cm. pressure nearly all of the Kupffer cells have bizarre shapes and are dead, and many of them show shredding. Washing at 30 cm., immediately after the blood removal, has proved most favorable in yield both for numbers and for proportion of living cells.

The morphology of the Kupffer cells is the same whether they have been washed out with Tyrode or with pooled homologous serum; and those of the rabbit and the dog have the same general features. Their size is largely conditional on how much particulate matter they contain. Those having little are from 30 μ to 40 μ across when spherical, whereas those crammed with particles may be 100 μ in diameter. Very occasionally one is seen with no particles, as rarely indeed as in the washings from normal livers. The cells that have taken up little or no material show a granular cytoplasm in which lie nucleus, vacuoles, and particulate matter, surrounded by a thick layer of transparent cytoplasm. The two sorts of cytoplasm grade into each other in the case of rabbit phagocytes, whereas in those of the dog the clear outer layer is as sharply demarcated as the rind of an orange. If the cells die while in the spherical form the granular cytoplasm often comes to lie at one side like a crescent moon, with the "old moon" of clear cytoplasm in its arms (Fig. 1). The nucleus is in the middle of the crescent, with the ink or iron particles to either side. Under such circumstances one sees that the proportion of transparent cytoplasm is great.

Within a few minutes after living Kupffer cells containing a moderate number of particles have settled on a glass surface at room or body temperature a zone of clear cytoplasm is seen to be extending out around some of them (Figs. 1 and 3) and they slowly flatten, the granular cytoplasm yielding last. Soon this lies in the midst of a great circular sheet of pellucid, glassy, apparently structureless material (Fig. 1, and Fig. 1 of the accompanying paper). None of the pictures that we have been able to obtain of this membrane gives a just idea of its proportions, or of its smoothly curving, circular outline. Always it has been retracted partially and irregularly as result of the stimulus of light. During the outward extension of the membrane, lava-like flows can be seen on its surface, when the light is cut down, and at its edges fimbriated or "petaloid" extrusions, at times appearing whip-like, which are in constant slow motion. Their edges can easily be mistaken for flagella if resolution with the microscope is poor. They are still present and active after the membrane has been completely extended. It then appears almost perfectly round, the slightly raised central hummock of granular material at its center making up only one-fourth

to one-third the entire diameter of the cell. This may amount to 150 μ or more, but usually from 40 μ to 80 μ with an average of about 60 μ. Before the granular portion of the cell has flattened it looks, under the low power, like a black or brown specked marble in the center of a clear disc of glass,—the specks being the phagocyted material and the disc the membrane. The greater the amount of material that has been phagocyted the less proportionately is the amount of membrane. Its refractility is so slight that pictures of it are difficult to obtain. In homologous serum it can scarcely be seen.

Cells that have crammed themselves with particles are more frequently dead when washed out. Only a thin, pellucid skim of cytoplasm can be seen when they are in suspension; but living ones soon flatten out after they have settled on a glass surface, and then one sees that they have the same general characters as the others, but only a narrow outer zone of clear cytoplasm. They segregate neutral red promptly, showing that their immense burden of foreign matter has not "blocked" them, in this respect at least.

The nucleus of the Kupffer cell is large, oval, and eccentric, its vacuoles of highly various size up to that of an erythrocyte, scattered irregularly through the granular cytoplasm (Fig. 3). The cells promptly segregate neutral red in supravital preparations, then varying in color from reddish orange to a slightly crimson red. Some of them contain small phagocyted particles, but most of these lie separate in the cytoplasm, which occasionally contains leukocytes or red cells.

It will be seen that the general characters of the Kupffer cells place them with the clasmatocytes. They have an evident relation to the giant cells encountered with them. In fixed smears in serum, which have been colored with Wright's stain, one sees that both have a light blue cytoplasm and their nuclei a darker reticulation; but the nucleus of the Kupffer cell is oval and relatively dense whereas those of the giant cell are spherical and much larger, with an open network so that they appear spongy. The giant cells contain relatively little particulate matter. One can recognize potential ones by these features while they are still mononuclear (Fig. 4), and can find all gradations between them and Kupffer cells on the one hand and immense multinucleate elements on the other (Fig. 2).

In washings obtained from the liver at the time when the yield of

Kupffer cells is greatest, some are found in process of division. Paired cells joined by a flat face across a region of constriction are fairly frequent, and there are occasional tetrads and even larger masses. The cells of any such pair or group are of about the same size and contain about the same amount of particulate matter, as is not usually the case with individuals that have accidentally stuck to each other. We have several times observed paired cells lying within a single membrane, and in cultures have noted division by fission within a membrane which as yet appeared homogeneous. The great variation in size and particle content of the individual Kupffer cells is doubtless referable to their relative opportunity for phagocytosis and to the vicissitudes of proliferation.

Comparison with the Phagocytic Monocytes from the Liver

The discrimination of Kupffer cells from the phagocytic monocytes present with them in greater or less number in liver washings is ordinarily easy, the monocytes having the characteristic indented or saddle nucleus and many little granules of even size located in a central *"Hof,"* all taking the same color with neutral red. They are much the smaller cells, their diameter in the dog averaging only about one-third that of the Kupffer cells when both are in spherical form. In the rabbit this difference is less marked. Practically all of the Kupffer cells contain phagocyted particles whereas most of the monocytes have none, and the generality of the phagocytic ones but little, which is true as well of those found in exudates and in scrapings of the spleen. On the other hand they are far more actively motile than the Kupffer cells. They put forth a relatively small, circular membrane, and nearly all possess mitochondria staining with Janus green, few being found in the Kupffer cells.

A statistical listing of the features of the individual mononuclear phagocytes encountered in neutral red preparations from liver washings has disclosed rare intermediate elements having some of the characters of the monocyte and others of the Kupffer cell (clasmatocyte). The latter is known to be a specialized derivative of the endothelium of the liver sinuses, all stages in its differentiation being seen in fixed preparations of the hepatic tissue. Hence the question has arisen of whether some of the monocytes at least may not represent transition

forms from the endothelial syncytium. The evidence would appear to negate this possibility. The number of "transition forms" is never considerable, being no greater than in exudates containing both clasmatocytes and monocytes. The longer the liver is washed the greater is the preponderance of Kupffer cells over monocytes amongst the elements coming away. The change should be in the opposite direction if the monocytes represented early stages of differentiation from the endothelium, since they should be more difficult to dislodge than the Kupffer cells which are more or less sessile upon it. Portions of the endothelial syncytium as such never appear in the washings. Fragments of the well-washed liver, examined in some cases after enough digestion with weak trypsin to dislodge the phagocytes without killing them, have been found to yield many that are of clasmatocytic type, few that are monocytic, and no transition forms. The occasional monocyte much larger than those of the blood, rivalling the Kupffer cell in diameter, has always a large content of phagocyted matter, thus accounting for its size.

Many authors have described monocytic accumulations in and about the liver sinuses of animals injected with bacteria, foreign proteins, and other material. The considerable literature has been recently reviewed by Swift (4). De Haan and Hoekstra (5) injected, into a mesenteric vein of rabbits, monocytes derived from peritoneal exudates and marked by a content of trypan blue. They found some of these cells later, living along the liver sinuses; and in consequence they advanced the view that the Kupffer cells are merely a monocytic colony derived from the blood. Our findings render this view untenable. The Kupffer cell, as washed from the liver, has the typical characters of a clasmatocyte, and is far larger than any blood cell.

Comparison of Kupffer Cells with Other Reticulo-Endothelial Elements

The Kupffer cell is generally supposed to be essentially identical in its characters and functions with the clasmatocytes present in other organs, notably spleen and bone marrow. Some authorities go so far as to suppose that it may be merely a migrant from the spleen. We have found that the yield of Kupffer cells is not less than usual when rabbits have been splenectomized prior to the injections of ink or

iron;[1] while furthermore the cells have morphological features that distinguish them from splenic clasmatocytes. The cells obtained by scraping the splenic pulp of rabbits several times injected with India ink or with alien red cells have been studied both in Tyrode and in serum, by the inversion technic. Cunningham, Sabin, and Doan (6) encountered two types of phagocytes in splenic scrapings, the one monocytic and only about 15 μ in diameter, the other obviously clasmatocytic and sometimes measuring 30 μ. These latter are as large as some Kupffer cells; but we find that in the warm box they put forth a membrane on one side only, a broad, irregular tongue of cytoplasm like ground glass, never having an area larger than that of the granular, vacuolated portion of the cell. Injections of ink and of ferromagnetic iron oxide into the peritoneal cavity of rabbits results in exudates containing many clasmatocytes which put forth a membrane similar to but in general smaller than that of the Kupffer cells. Sabin, Doan, and Cunningham in their detailed description of the clasmatocyte of peritoneal exudates in the rabbit (7) make no mention of any such extensive membrane as that of the Kupffer cell.

Peculiarities of the Kupffer Cell

Except for the immense circular membrane, no feature of the Kupffer cell is more striking than its stickiness,—which is equally remarkable whether the enveloping medium is serum or Tyrode solution. It has much interfered with the study of the cells *in vitro*. Whatever touches their surface tends to adhere, whether it be red or white cells or "bodies" that are floating by; and hence in selecting them from the washings with the magnet some of these elements are inevitably included in the collection. If the liver circulation is interrupted for a few minutes prior to the washing out, many of the Kupffer cells coming

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[1] The production of hemochromatosis in rabbits by daily transfusions of rabbit blood over long periods of time results not only in the characteristic pigmentation of the liver parenchyma with hemosiderin but in pigmentation of the splenic clasmatocytes and Kupffer cells as well (Rous, Peyton, and Oliver, J., *J. Exp. Med.*, 1918, 28, 629). In order to learn whether the latter had perhaps migrated from the spleen this organ was removed from a number of rabbits prior to the transfusion period. When they were examined, after 3 to 6 months of transfusions, the same liver findings were obtained as in animals still possessing the spleen.

away will be covered with leukocytes which cannot be dislodged. When they contain ink or iron they sediment rapidly out of suspension and they soon become fixed so firmly on the bottom of the container that a considerable proportion resist even a forcible jet of Tyrode. Always some loss occurs from this cause on the sides of the taper flask during selection with the magnet, even though the suspension is frequently agitated; and there is a more considerable loss on the collodion membrane to which the cells are attracted. Centrifuging at the lowest speed that will bring them down in a few minutes in a flat-bottomed tube results in a lumping together that can be only partially broken up by vigorous pipetting; and despite it a thick skim of cells regularly remains on the glass. In slide and cover-glass preparations one frequently sees erythrocytes or leukocytes which had appeared merely to touch the extended membrane of a Kupffer cell, dragged across the field with this membrane when it retracts. A sticky surface is evidently one of the special characters enabling the cell to perform its task of phagocytosis. The stickiness of leukocytes from the blood and from exudates,—with both of which we have had much experience,—is in comparison negligible.

Kupffer cells are notably sensitive to injury, as evidenced by the number that die within a few minutes after they have been washed from the liver. Most of those procured from the rabbit survive only 4 or 5 hours in Tyrode at room temperature. They are rapidly killed by the slight alkalinity which develops in this fluid on storage in the ice box; and its reaction must be brought to pH 7.2–7.4 by bubbling CO_2 through it prior to flushing out the liver if the cells are to survive during the 2 to 3 hours required for selection with the magnet. When they are transferred immediately afterwards to serum in a culture flask many lie as if dead for a day or more before putting forth a membrane and beginning to move about. Kupffer cells of the dog are relatively resistant, many surviving for 24 hours in Tyrode at 4°C. and a few even for 48 hours, at which time, however, most of the monocytes and polymorphonuclears associated with them are still in excellent state.

The Kupffer Cells in Cultures

On *a priori* grounds it had been feared that most if not all of the Kupffer cells coming away in the washings would be over-mature or else rendered incapable by their content of foreign matter, with result that they would not long survive in cultures. This has not proved to be the case, though it is true that cells which have taken up coarse lumps from a poorly-ground preparation of iron oxide die very soon. Those that have taken up the material as fine particles flourish under proper *in vitro* conditions and proliferate, sharing said particles almost equally, with result that the individual cells soon come to contain but few.

The iron particles turn black when the cells die *in vitro*, but while these are doing well they remain yellowish brown, persisting without apparent change. By their appearance and response to the magnet they have been recognized within Kupffer cells washed from the liver 19 months after the injection into the blood stream, as stated in the paper on method (1).

On first removal to cultures in plasma or serum most of the Kupffer cells are still aggregated in coarse lumps which have resisted pipetting. These usually remain as such for a day or more and then the individuals composing them disengage themselves and move slowly away (Fig. 3 of the accompanying paper). The first step showing that the cell is alive is the extrusion of the characteristic large, clear, circular membrane. This happens only on a surface. The cell may remain where it is or begin to creep about very slowly, hours of observation being often required to discern a change in position. The petaloid edge of the membrane, though, is in constant, rather quick, wavy motion, with "lava flows" over its surface and slight, gradual withdrawals and re-extrusions. In even the thinnest plasma clot the cells are greatly hampered and move but little in the course of days, leaving in some cases a clear track behind them, doubtless of digested fibrin web. When moving they frequently elongate, becoming slug-shaped. They progress only along the glass, never penetrating into the over lying plasma layer. When they are about to divide the particles of iron become massed in approximately equal quantity on opposite sides of the cell, and occasionally are ranged in two roughly parallel

lines as if they were intrinsic elements.   As soon as division has oc-curred the cells move apart.   It is possible to maintain them for a few days within a thin layer of clot overlaid by serum, if it is frequently washed with Tyrode and the serum changed; but they tend to die off in a week or two, despite some initial multiplication, even when pro-teolytic products are furnished of the sort on which Baker has shown (8) that chicken monocytes thrive.

When transferred in serum to Carrel dishes containing lens paper the cells distribute themselves upon this, as already described, and cling so firmly that the serum can be pipetted off and replaced daily with but slight loss, more than compensated for by proliferation.   A few of the cells coming away at the daily replacement are dead; but with neutral red most are found to be in good condition, an incidental wastage.   When the cultures are crowded and the cells contain much iron or ink, some secondary formation of giant cells may take place; but the general tendency of the Kupffer cells is to scatter and live apart (Fig. 5 of the accompanying paper).   This tendency is curiously instanced when they mount from the bottom of the dish along a fibre of lens paper.   They then space themselves on the fibre at almost regular intervals, with no clustering back of an obstruction or clam-bering over one another (Fig. 6 of the accompanying paper).   Yet despite what would appear to be a negative chemotaxis as concerns one another, the cells do poorly and soon die off when widely separated, like those of the other kinds thus far cultivated.   Efforts to prevent this by regulating the pH with a mixture of nitrogen, oxygen, and carbon dioxide have proved unavailing.

When living on strands of lens paper, the Kupffer cells lie with mem-brane spread, and the edges of its glassy expanse can be discerned only imperfectly (Fig. 6).   It thickens somewhat toward the center and here the granular cytoplasm containing nucleus, vacuole, and partic-ulate matter projects as a mound or lump or more or less tangential sphere (Figs. 5 and 6).   The cells appear stationary but prolonged observation shows that they move slowly along the fibre.   When this is very slender they may wrap themselves about it, then appearing spindle-shaped.   Only occasionally is a cell found to be connected by a protoplasmic process with a neighboring fibre (Figs. 7 and 8). These facts, the difficulty of dislodging normal Kupffer cells from the

liver, their tendency to spread like pancakes without extrusion of pseudopods worthy the name, all lead one to doubt the current view that many of them normally lie athwart the blood current, moored to the sinus walls by slender protoplasmic processes. This view is based on fixed, and perhaps distorted, histological preparations.

In every magnet collection of Kupffer cells some monocytic elements containing iron are to be found as well. Phagocytes of this sort generally contain but little and in consequence most of them escape the magnet, the proportion being much reduced from that present in the washings: sometimes almost none reach the cultures with the Kupffer cells, and again they contribute one-fourth to one-fifth of the cell population. They survive as such during the first days of life *in vitro* and can be readily recognized. Their ultimate fate is not known. A single cell type, the result of repeated division, is found in cultures that have been propagated for a week. This cell type contains little or no iron,—for the particles have been shared by the multiplying elements; it is not quite so large as the "adult" Kupffer cell, though possessed of a similar membrane and nucleus; and the granules are smaller and not so various as in this latter, though they are still of unequal size. In other words the general characters of the clasmatocyte have been retained, though they are not as pronounced as in the original Kupffer cell. We have in general discarded the cultures before they have reached this stage.

The iron-containing giant cells which come over into the cultures on lens paper live to all appearance unchanged (Fig. 9). They tend to fix themselves where many fibres touch one another, spreading out irregularly in various directions; but not infrequently they change place, gliding along a fibre, which they envelope, as if it were a wire. When on the bottom of the dish, giant cells containing little iron put out a membrane even larger than that of a Kupffer cell; but those stuffed with the foreign material have no more than enough pellucid cytoplasm to cover the great mass of granular cytoplasm with a thin coat. Such crammed cells remain roughly spherical while fixed on a fibre.

SUMMARY

The Kupffer cells procured from the liver of the rabbit and dog for culture *in vitro* have the typical characters of clasmatocytes. They are readily discriminated from the monocytes washed from the liver with them; and they have certain peculiar features which suffice to differentiate them from some at least of the clasmatocytes of other organs. Their surface is extraordinarily sticky,—far more so than that of blood leukocytes or of the clasmatocytes found in peritoneal exudates; and in consequence they are exceedingly difficult to handle *in vitro*. They put forth enormous, pellucid, circular membranes resembling those of exudate clasmatocytes but larger. Splenic clasmatocytes, on the other hand, put forth rather small, one-sided ground-glass membranes like broad tongues. On comparing them with Kupffer cells and exudate clasmatocytes one perceives that they are not wholly identical in their characters, but have secondary peculiarities. However, there exist good morphological reasons for grouping them together and terming them all reticulo-endothelial.

Kupffer cells are notably sensitive to injury, surviving in Tyrode solution for a much shorter time than blood leukocytes. However, they can be readily cultured on lens paper in serum. Under such circumstances they scatter on the fibres and live separately, presenting the same general aspect as when in the liver; but in the course of proliferation they soon lose some of their pronounced characters, retaining such as are common to clasmatocytes in general.

A considerable population of ordinary leukocytes exists in the hepatic sinuses over and above those circulating in the blood. During infection, their number may greatly increase. Several cubic centimeters of packed white cells have been obtained from the liver of a sick dog. The fact has been realized that leukocytes may stop a while in the liver, yet the extent of the accumulation which sometimes takes place seems deserving of stress.

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EXPLANATION OF PLATES

PLATE 43

FIG. 1. Washing from the liver of a rabbit injected intravenously with India ink repeatedly: fresh slide and cover-glass preparation. Two Kupffer cells are putting forth membranes along the slide surface, but these have retracted irregularly as result of the strong light. A third Kupffer cell is dead. The ink in it lies in a crescent at one side of an "old moon" of pellucid cytoplasm. The nucleus is midway in the crescent. × 350.

FIG. 2. Giant cell washed from the liver of a rabbit repeatedly transfused with incompatible rabbit's erythrocytes and injected intravenously with India ink shortly before the washing. The many large, rounded nuclei lie scattered in a granular cytoplasm containing ink, red cell debris, and phagocyted white cells. Wright's stain. × 650.

FIG. 3. Rabbit Kupffer cell containing ink particles, and stained with neutral red; slide and cover-glass preparation of fresh liver washings. The black particles are the ink, the gray spots dye-stained vacuoles. The membrane is as yet only half extended. To render it visible the photograph has been taken with the light coming from one side. The cell is of great size as compared with the lymphocyte and red cells associated with it in the washings. × 1000.

FIG. 4. Another portion of the material furnishing Fig. 2, colored with Wright's stain to show a mononuclear giant cell. The nucleus of this cell is large, circular, and spongy in appearance, and it has taken up but little ink. The arrow points to the oval, dark nucleus of a neighboring Kupffer cell so loaded with ink particles that it ruptured when the preparation was made. × 950.

PLATE 44

FIG. 5. Side view of an iron-containing dog Kupffer cell fixed on a lens paper fibre; 3rd day of culture. The resemblance is complete to cells of the same sort as seen on the walls of liver sinuses in stained sections of the organ. × 270.

FIG. 6. Dog Kupffer cells living on lens paper. The arrow points to the edge of a cell membrane. The dark particles are intracellular iron oxide. × 400.

FIG. 7. Dog Kupffer cell living on lens paper, with a process attached to a neighboring fibre. × 400.

FIG. 8. Another cell with such a process, as also some cells projecting into the serum like hemispheres. The dark particles are intracellular iron oxide. × 400.

FIG. 9. Iron-containing giant cell from a dog liver; 4th day of culture. × 400.

PLATE 43

(Bear Land Roust Characters of Kupffer cells in vivo)

(Bronfenbrenner: Characters of Kupffer cells in vivo)

EXPERIMENTAL TYPE III PNEUMOCOCCUS PNEUMONIA IN MONKEYS

I. Production and Clinical Course

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PLATES 45 TO 47

Blake and Cecil (1) were the first investigators who regularly produced experimental lobar pneumonia in monkeys. *Macacus syrichtus* was used in the majority of experiments, while *Cebus capucinus* and *M. rhesus* were found to be less satisfactory. In most instances, Type I Pneumococcus was employed; in the three cases in which pneumonia was produced by the inoculation of Type III Pneumococcus, spontaneous recovery occurred. Schöbl and Sellards (2) obtained similar results with Type I Pneumococcus in the same species of animal. Stuppy, Falk, and Jacobson (3) attempted to produce pneumonia with Type I Pneumococcus in monkeys of the *M. rhesus* and *C. capucinus* species, but found animals of these species to be resistant to infection, and the authors therefore considered them unsuitable for such an experimental study.

The present study was begun as an effort to induce experimental lobar pneumonia with Type III Pneumococcus in monkeys. Dubos and Avery (4) described, in 1931, an enzyme of bacterial origin which possessed the capacity of decomposing the type-specific capsular polysaccharide of Type III Pneumococcus *in vitro*. The enzyme was shown, in addition, to have a distinct therapeutic action upon Type III pneumococcus infections in mice and rabbits (5–7). Consequently, it was thought that if experimental Type III pneumococcus pneumonia could be successfully produced in monkeys, a study of the effect of specific enzyme in the therapy of this infection could be made. The scope of this paper, however, is limited to the production of experimental pneumonia with Type III Pneumococcus in monkeys.

Materials and Methods

1. Experimental Animal.—The Java monkey (*M. cynomolgos*) was used throughout the study. In several ways the choice was fortunate, for animals

TYPE III PNEUMOCOCCUS PNEUMONIA. I

animals were quite sick, it was sometimes possible to obtain only small amounts of blood, and rapid clotting was a disturbing feature.

(d) *Symptoms.*—The animals were observed closely for degree of activity, appetite, strength, cough, character of respirations, and thoracic tenderness.

(e) *Diet.*—The monkeys were fed banana, orange, and bread and milk. During the period of illness they not infrequently refused the standard diet but accepted substitutes such as greens, carrots, prunes, and water. Diarrhea was an infrequent occurrence.

(f) *X-Rays.*—Roentgenograms of the chest were made before infection and at least once daily during the course of the disease.

(g) *Autopsies.*—Performed under sterile conditions as soon as possible after death. Frequently, the trachea was clamped before the chest was opened. Cultures were made of the heart's blood, of pleural and pericardial fluid, and of the pulmonary lobe chiefly involved. Stained preparations of the pneumonic exudate were sometimes examined for evidence of phagocytosis. At times the lungs were inflated with air. The heart and lungs were removed *in toto.*

TABLE I

Mortality in Experimental Type III Pneumococcus Pneumonia in Monkeys*

Diagnosis	No. of animals	No. recovered	No. died	Mortality
				per cent
Pneumonia without septicemia.............	20	20	0	0
Pneumonia with septicemia (1–250 colonies per cc.)........	20	11	9	45
Pneumonia with septicemia (250–2000 per cc.)........	12	3	9	75
Pneumonia with septicemia (2000 or greater)..	16	0	16	100
Total........	68	34	34	50

* Classified on the basis of height of septicemia in first 3 days.

RESULTS

This report comprises an analysis of the data obtained in 68 monkeys, in which the experimentally induced Type III pneumococcus pneumonia was allowed to run its course without therapeutic interference. In the entire group, the mortality rate was 50 per cent. In Table I, the series is divided into groups on the basis of the height of the septicemia present during the first 3 days after infection. In animals in which pneumonia occurred without demonstrable septi-

cemia, recovery invariably resulted.   It can readily be seen, however, that in the presence of septicemia the mortality rate rises progressively as the number of organisms in the circulating blood increases.   In Chart 1 are shown the height of septicemia in the first 3 days after
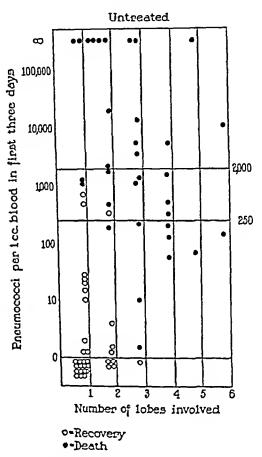
Untreated

o=Recovery
•=Death

CHART 1. The relation of the height of septicemia and the amount of pulmonary involvement to the outcome of the disease.

infection and the number of pulmonary lobes involved during the disease in relation to the outcome of the disease.   In the majority of animals which recovered, one or part of one lobe was involved, and in only one instance were three lobes affected.   In all but one

TABLE II

Experimental Type III Pneumococcus Pneumonia in Monkeys

No.	Weight	Dose	Route*	Date		Days after infection							Remarks
						1	2	3	4	5	6	7	
	gm.	cc.				Pneumonia without septicemia							
5	2725	0.5	i.t.	11-10-31	Bl. cult.† X-ray‡ WBC§	0 1/2 RLL	0 Same	0 Same	0 Same		0 Same	0 Same	Recovered 4th day
6	1575	0.5	i.t.	11-17-31	Bl. cult. X-ray WBC	0 1/2 RLL	0 Spread	0 Clearing	Clearing				Recovered 3rd day
1-3	950	0.5	i.t.	1-12-32	Bl. cult. X-ray WBC	0 1/2 RML	0 Spread	0 Clearing	0 Clearing				Recovered 3rd day
1-5	1450	1.0	i.t.	1-19-32	Bl. cult. X-ray WBC	0 1/2 RLL	0 3/4 RLL	0 RLL	0 RLL	0 Clearing		Clearing	Recovered 3rd-4th day
2-5	1775	0.2	i.t.	2-15-32	Bl. cult. X-ray WBC 10.8	0 1/3 LLL 21.3	0 1/3 LLL 14.3	0 Clearing LLL New in RLL 6 5	0 Clearing 10 5				Recovered 4th day
3-2	1500	0 3	i.t.	3-21-32	Bl. cult. X-ray WBC 10 0	0 1/3 LLL 9 4	0 Spread 13 0	0 Clearing	0 9.2	0			Recovered 3rd day
3-7	2290	0 4	i.t.	4-18-32	Bl. cult. X-ray WBC 24 2	0 1/3 RLL 21 2	0 Spread 13.2	0 1/2 RLL 13.2	0 Spread 14.0	0 3/4 RLL 16.5		0 RLL 16.2	Recovered 7th day

No.	Wt.	Dose†	Route*	Date	Test							Remarks
4-4	1825	0 4	i.t.	5-2-32	Bl. cult.	0	0	0	0	0		Recovered 3rd day
					X-ray	1/2 RLL	Same	Same	Clearing			
					WBC	25 8	9.7	5.4	6.0	6.0		
6-4	1250	1 0	i.t.	8-2-32	Bl. cult.	0	0	0				Recovered 4th day X-ray: Mottled density 1/2 RML and RUL
					X-ray	Mottled	Mottled	Confluent	Clearing			
					WBC	17 0						
7-1	2450	0.1	i.b.	10-17-32	Bl. cult.	0	0	0	0			Reenvered 3rd day
					X-ray	1/3 RLL	2/3 RLL	Clearing	Clearing			
					WBC	26.9	16.6	10.2				
7-9	2400	0.25	i.b.	12-12-32	Bl. cult.	0	0	0	0	0		Recovered 3rd–4th day
					X-ray	1/3 RLL	Same	Clearing	25.6	28.2		
					WBC	21.2	11.3	14.6				
8-3	1950	0 25	i.b.	12-19-32	Bl. cult.	0	0	0	0	0		Recovered 4th day
					X-ray	1/3 RLL	2/3 RLL	Same	Clearing	20.8		
					WBC	31 6	35.7	25.8	15.1			
8-9	2000	0 3	i.b.	1-16-33	Bl. cult.	0	0	0	0	0	Same	Recovered 12th day X-ray: RUL cleared as RLL became consolidated
					X-ray	1/2 RUL	Spread	RUL	Same	Dense		
					WBC	15 7	27.5	12.5	11 1	22.3		
9-3	2125	0 3	i.b.	1-30-33	Bl. cult.	0	0	0	0	0	0 RUL	Recovered 8th day X-ray: RML cleared as RUL became consolidated
					X-ray	1/3 RML	Spread	Spread	RML	28.3		
					WBC	21 5	11 6	30 2	11.2			
1-08	1500	0.3	i.b.	1-21-33	Bl. cult.	0	0	0	0	Clearing		Recovered 3rd–4th day Irregular slight mottled density midportion right lung
					X-ray	Mottled	Same	Same	Same			
					WBC	33 9	17 0	28.0	16.8			

* i.t. = intratracheal inoculation; i.b. = intrabronchial inoculation.
† Number of pneumococci obtained in poured plate culture per 1 cc. of blood; + = growth occurred in broth cultures of blood; C = contaminated.
‡ RUI, RMI, RLI, LUI, LMI, LLI = right upper, right middle, right lower, left upper, left middle, left lower lobes, respectively. (Cardiac lobe not included.)
§ White blood cells in thousands per c.mm. of blood.

TABLE II—Continued

No.	Weight (gm.)	Dose (cc.)	Route*	Date		1	2	3	4	5	6	7	Remarks

Pneumonia without septicemia—Concluded

No.	Weight	Dose	Route	Date		1	2	3	4	5	6	7	Remarks
1-15	1500	0.4	i.b.	5-15-33	Bl. cult. X-ray WBC 17.3	0 1/3 RLL 18.4	0 1/2 RLL 26.5	0 Same 11.5	0 Clearing 13.5				Recovered 3rd-4th day
1-16	1800	0 4	i.b.	5-15-33	Bl. cult. X-ray WBC 21.1	0 RML 40.6	0 Spread 31.0	0 Clearing 18.1	0 Clearing 13.7				Recovered 3rd day
1-21	1800	0.45	i.b.	5-23-33	Bl. cult. X-ray WBC 20.8	0 1/3 RUL 29.7	0 Same 23.5	0 Clearing 14.6	0 Clearing 23.5				Recovered 3rd day
1-22	1750	0.45	i.b.	5-28-33	Bl. cult. X-ray WBC 31 0	0 2/3 RUL 41.7	0 Same 50.0	0 Clearing 24.8	0 Clearing 23.7				Recovered 3rd day
1-25	1750	0 45	i.b.	5-29-33	Bl. cult. X-ray WBC 20.6	0 1/3 RUL 21.2	0 Spread 13.0	0 RUL 9.5	0 Clearing 13.9	Clearing			Recovered 4th day

Pneumonia with septicemia (1-250 colonies per cc. in first 3 days after infection

No.	Weight	Dose	Route	Date		1	2	3	4	5	6	7	Remarks
2	1650	0.5	i.t.	10-26-31	Bl. cult. X-ray WBC	0 1/2 RLL 1/2 RML	15 Spread	4 3/4 RLL RML	0 Clearing				Recovered 4th day
7	3180	1.0	i.t.	11-28-31	Bl. cult. X-ray WBC	0 1/2 LLL	0 LLL	1 LLL	0 Same	0 Clearing			Recovered 4th day

						Obs.							Remarks
1.2	1390	1.0	1t	12-16-31	Bl. cult. / X-ray / WBC	0 LLL	0 LLL	0 Same	0 Same	0 Same	0 Same	LLL 1/3 LUL	Regarded as recovered. Sacrificed 6th day Autopsy: Pneumonia LLL, 1/3 LUL III culture: Pn. III
5.8	2000	0.75	l.t.	7-12-32	Bl. cult. / X-ray / WBC 26.3	0 1/3 LLL / 41.6	2 1/2 LLL / 9.4	6 LLL / 4.3	0 LLL 1/2 LUL / 7.1			LLL LUL	Recovered 4th-5th day. Sacrificed 7th day Autopsy: Pneumonia LLL (beginning resolution), 2/3 LUL (grey)
6.0	2100	0.85	l.t.	7-27-32	Bl. cult. / X-ray / WBC 13.0	0 1/3 LLL / 13.1	20 3/4 LLL / 7.5	0 Same / 11.7	0 Clearing / 13.0				Recovered 4th day
4.7	2200	0.5	l.b.	9-12-32	Bl. cult. / X-ray / WBC 20.6	1 1/2 RLL / 13.1	— Same / 8.8	1 Same / 10.6	0 / 9.3				Recovered 4th day
4.5	2050	1.0	l.b.	9-22-32	Bl. cult. / X-ray / WBC 20.4	0 1/3 RLL / 52.8	23 RLL / 21.3	6 / 21.0	5 Denser / 25.3	0 Same / 23.3			Recovered 5th day
9.7	2700	0.33	l.b.	2-20-33	Bl. cult. / X-ray / WBC 27.9	0 1/2 RUL / 47.0	RUL 1/3 RML / 45.0	2 RUL 1/2 RML / 30.9	10 Same / 28.0	4 Clearing / 17.8	1 / 25.6	0 Clearing / 22.6	Recovered 6th day
1.07	1750	0.3	l.b.	1-21-33	Bl. cult. / X-ray / WBC 19.6	0 1/2 RLL / 12.3	0 1/2 RLL / 10.8	10 Spread / 9.3	0 Clearing / 11.1	0 Clearing / 22.4			Recovered 4th day
1.28	1500	0.5	l.b.	6-5-33	Bl. cult. / X-ray / WBC 16.2	1 1/3 RLL / 24.8	6 1/2 RLL / 19.2	27 RLL / 10.2	25 Same / 14.3	2 Clearing / 70.0		0 / 20.0	Recovered 6th day 8th day Autopsy: Pneumonia RLL resolving
1.32	1650	0.5	l.b.	6-5-33	Bl. cult. / X-ray / WBC 13.5	0 1/3 RLL / 61.6	3 Spread / 38.6	2 1/2 RLL / 21.8	0 Clearing / 10.6	Clearing			Recovered 4th day

TABLE II—Continued

Pneumonia with septicemia (1–250 colonies per cc. in first 3 days after infection)—*Concluded*

No.	Weight gm.	Dose cc.	Route	Date		Days after infection 1	2	3	4	5	6	7	Remarks
4	2150	0.5	i.t.	11-4-31	Bl. cult.	12	+	185		653	1728	+	Died 7th day. Autopsy: Pneumonia RLL, RML. Injection lower margin RUL
					X-ray	1/2 RLL	RLL	Spread		Denser	RLL RML	Same	
					WBC								
1.0	1650	1.0	i.t.	12-9-31	Bl. cult.	+	+	+					Died 5th day. Autopsy: Pneumonia 1/2 RLL, 1/2 LLL, most of LML and LUL
					X-ray	1/2 RLL Lt. hilus	RLL Lt. hilus	Spread					
					WBC								
1-1	1300	1.0	i.t.	12-9-31	Bl. cult.	10	0	+	—	+			Died 5th day. Autopsy: Pneumonia LLL, LML, 1/2 LUL
					X-ray	2/3 LLL	LLL	LLL LML	—	LLL LML 1/2 LUL			
					WBC								
1.6	1275	1.0	i.t.	1-19-32	Bl. cult.	6	133	38	37				Died 5th day. Autopsy: Pneumonia RML, 2/3 RUL, 2/3 LLL (mottled). Culture: Pericardial fluid = Pn. III
					X-ray	1/3 RLL	2/3 RLL 1/3 RML 1/2 RUL	RLL RML 1/2 RUL	Spread				
					WBC								
1.7	1250	1.0	i.t.	1-26-32	Bl. cult.	—	140 Post mortem			3500 Post mortem			Died 2nd day. Autopsy: Left empyema; pneumonia, all lobes extending from right and left hilus. (Included here because of p. m. culture)
					X-ray WBC 20.0	Diffuse				Spread			
4-1	1975	0.4	i.t.	4-26-32	Bl. cult.	1							Died 5th day. Autopsy: Empyema, right and left. Pneumonia LLL, LML, 2/3 RUL, RLL, 1/2 RML, RUL (patchy)
					X-ray	1/3 RLL 1/3 LLL							
					WBC 20.7	26 0							
						4 Spread 2.2	75 Spread 3.2	1800 Spread 1.7					

Pneumonia with septicemia (250–2000 colonies per cc. in first 3 days after infection)

							0 Rt. hilus Lt. hilus	22 3/1 RLL Lt. hilus	60 Spread	+ RML RUL 2/3 RLL	30 Same	26 Spread	
5-2	1475	t 0	1t	6-9-32	Bl. cult. X-ray	WBC 17 7	26 6	10 4	10 1	29 1	33.9	20 0	Died 6th day. Autopsy: Pneumonia 2/3 RLL, RML, 1/2 RUL, 2/3 LUL. Culture: Pericardial fluid = Pn. III
9 0	1950	0 3	1h.	1-23-33	Bl. cult. X-ray	WBC 23 2	33 2/3 RLL 13.5	32 2/3 RLL 27 0	236 Same 10 5	171 Spread 11.7	106 RL 18 0	1200	Died 8th day. Autopsy: Right fibrinopurulent pleurisy, pericarditis; pneumonia RLL, RML, 1/2 RUL
4-10	2550	0 3	1h.	4-21-33	Bl. cult. X-ray	WBC 11.1	0 1/2 RLL 17.1	0 RLL 9.5	2 RLL RML 2.5	18 RLL RML 1/2 LLL 12.2			Died 5th day. Autopsy: Pneumonia RLL, RML, 1/2 LLL, congestion RUL, bilateral maxillary sinusitis

							208 1/2 RLL	700 Same	580 Spread	78 RLL	50 Same	352 Denser	72 Clearing	
2-7	1110	0 2	lt.	2-19-32	Bl. cult. X-ray	WBC 13 5	40.1	3.5	6.8	8.5	13.5	6.8	5.1	Recovered 9th day. Sacrificed 10th day. Pneumonia RLL. Early resolution. Cultures sterile
6 6	3550	2.0	lt.	8-16-32	Bl. cult. X-ray WBC 21.6	468 RLL 40.1	61 Same 23.0	172 Denser 15.1	0 Same 12.1	106				Recovered 4th day. Sacrificed 5th day. Autopsy: Pneumonia RLL
1-27	1700	0.5	1h.	6-5-33	Bl. cult. X-ray WBC 21.0	110 2/3 RUL 40.0	36 Spread 11.1	366 RUL RML 13.7	2 Same 6.8	0 Clearing 31.0		Clearing		Recovered 5th day. Sacrificed 8th day. Autopsy: RUL resolving; RML, early resolution
1-1	1150	1.0	1.t.	1-12-32	Bl. cult. X-ray WBC	+ 1/2 RLL	C Spread	+ RLL RML	RLL RML					Moribund. Sacrificed 4th day. Autopsy: Pneumonia RLL, RML. Pericarditis (hem. strep.) IIII culture: Pn. III and hem. strep.

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TABLE II—Continued

No.	Weight gm.	Dose cc.	Route*	Date		Days after infection							Remarks
						1	2	3	4	5	6	7	
					Pneumonia with septicemia (250–2000 colonies per cc. in first 3 days after infection)—*Concluded*								
2-0	1400	1.0	i.t.	2-8-32	Bl. cult.	0	1600	+					Died 3rd day Autopsy: Calcified cysts lungs and abdomen. Pneumonia RLL, RML, center RUL, HB culture: Pn. III and Gram-neg. bacillus.
					X-ray	1/2 RLL	RLL RML	RLL RML 1/3 RUL					
					WBC 16.0	5.3	3.4	2.3					
2-8	1875	0 3	i.t.	3-7-32	Bl. cult.	12	64	460	+				Moribund, sacrificed 5th day Autopsy: Fibrinopurulent pleurisy right; pericarditis; pneumonia RLL, RML, RUL, part LLL
					X-ray	1/2 RLL	2/3 RLL	RLL RML	RLL RML RUL	Same			
					WBC 23.0	18.5	8.0	4.2	13.5				
6-1	2625	1 0	i.b.	10-4-32	Bl. cult.	36	224	1440	2400				Died 5th day Autopsy: Pneumonia RLL, RML, LML
					X-ray	1/3 RLL	2/3 RLL	RLL RML 1/3 LML	RLL RML LML				
					WBC 17.2	24.1	15.0	14.6	10.6	8			
6-3	2300	0 05	i.b.	10-10-32	Bl. cult.	0	19	1812	2240	4240			Died 5th day Autopsy: Right fibrinopurulent pleurisy; pericarditis; pneumonia RLL
					X-ray	1/3 RLL	3/4 RLL	RLL	Same	Same			
					WBC 52.0	31 2	20.9	15.7	29.7	36.9			
7-0	3750	1 0	i.b.	10-25-32	Bl. cult.	43	360	1208	8				Died 5th day Autopsy: Pneumonia RLL HB culture: Pn. III and Gram-neg. bacillus. Pn. III Pericardial fluid:
					X-ray	1/3 RLL	RLL		RLL				
					WBC 14.6	13.8	7.6	7.6	11.0	8.3			

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No.			Route	Date	Test						Outcome
8-2	2600	0 25	i.b.	12 19-32	Bl. cult.	0	0	1320	∞	∞	Died 5th day Autopsy: Fibrinopurulent pleurisy; pneumonic abscess; 1/3 RLL containing abscess 1 x 0.5 cm.
					X-ray	1/1 RLL	2/3 RLL	2/3 RLL	Same	Less dense	
					WBC 9 1	13 9	19.8	25 8	4.9	2 6	
9 6	2600	0 31	i.b.	2-20-33	Bl. cult.	0	10	1220 RUL RML	∞ Pneumothorax left side		Died 4th day Autopsy: Fibrinopurulent pleurisy, right; pneumonia RUL. RML, 1/3 RLL.
					X-ray	1/3 RUL	3/1 RUL				
					WBC 18 2	22.3	20.2	23 6			
4 6	1975	1 0	i.b.	9-27-32	Bl. cult.	170	120	315	61		Died 5th day Autopsy: Pneumonia LLL, LML, 1/3 LUL, 1/3 RML
					X-ray	LLL	Spread	Same	LLL LML		
					WBC 19 3	7 6	6 2	1.7	2.6		

Pneumonia with septicemia greater than 2000 colonies per cc. in first 3 days after infection

No.			Route	Date	Test			Outcome
1	1150	1 0	l.t.	10-20-31	Bl. cult.	∞ RLL RML		Died 1st day Autopsy: Fibrinopurulent pleurisy; pericarditis; pneumonia RLL, RML.
					X-ray			
					WBC			
1 8	1350	1 0	l.t.	1-26-32	Bl. cult.	11,000 2/3 LLL, 1/2 LML, 1/2 LUL		Died 2nd day Autopsy: Pneumonia inner 3/4 LLL, central 1/2 LML, median 1/3 LUL.
					X-ray			
					WBC 17.0	17.7		
2-3	1350	1 0	l.t.	2-8-32	Bl. cult.	12,100 Both lungs diffuse, patchy		Died 2nd day Autopsy: Involvement of all lobes extending from hilus BB: Pn. III and Gram-neg. bacilli
					X-ray			
					WBC 17.7	10.8		
2-2	1100	0 1	l.t.	2-21-32	Bl. cult.	220 1/3 LLL, 1/3 RLL	∞ LLL LML RLL	Died 2nd day Autopsy: Pneumonia LLL, LML, RLL, part RML. BB: Pn. III and a few Gram-neg. bacilli
					X-ray			
					WBC 21.0	22.0	7.5	

TABLE II—*Concluded*

Pneumonia with septicemia greater than 2000 colonies per cc. in first 3 days after infection—Concluded

No.	Weight gm.	Dose cc.	Route*	Date		Days after infection							Remarks
						1	2	3	4	5	6	7	
2-6	1750	0.3	i.t.	2-29-32	Bl. cult.	340	1600	5280	2280				Moribund. Sacrificed 6th day. Autopsy: Pneumonia RLL, RML, RUL, inner 1/4 LLL
					X-ray	1/3 RLL 1/2 RML 1/2 RUL	Spread	Spread	RLL RML RUL	Spread 1/4 LLL			
					WBC	15.0	10.0	7.2	6.1				
3-5	1450	0.5	i.t.	4-1-32	Bl. cult.	240	1390	5600					Moribund. Sacrificed 4th day. Autopsy: Small empyema, left; pneumonia RLL, RML, 1/2 RUL
					X-ray	2/3 RLL 1/3 RML	RLL RML	RLL RML 1/2 RUL	Same				
					WBC 23 9	17.8	8.3	4.2					
3-6	1450	0.4	i.t.	4-11-32	Bl. cult.	6400	∞ RLL RML						Died 2nd day. Autopsy: Beginning empyema; pneumonia RLL, RML; congestion RUL
					X-ray	2/3 RLL							
					WBC 11.1	4.8	1.2						
5-4	1600	1.0	i.t.	6-22-32	Bl. cult.	∞ 1/3 RLL 1/2 LLL	∞ Spread						Found dead 3rd day. Autopsy: Pneumonia LML, 1/3 RLL; LLL, 1/2
					X-ray								
					WBC	14.1	1.2						
5-5	1950	0.75	i.t.	7-5-32	Bl. cult.	+ Diffuse on right	3360 Spread						Died 2nd day. Autopsy: Pneumonia RLL, 2/3 RUL, 1/2 RML; hilus LLL
					X-ray								
					WBC	7.5	3.1						

5.7	2235	1 0	l.t.	7-5-32	Bl. cult. X-ray WBC 18 3	+ 1/3 RLL 11.4	8736 2/3 RLL 5 7	20,800 RLL 1 4	RLL RML 5.0		Died 5th day Autopsy: Empyema; pneumonia RLL, RML. Pericardial fluid: Pn. III
5.9	2010	1 0	l.t.	7-10-32	Bl. cult. X-ray WBC 9 8	3010 Mottled shadow both lower lobes 8.0	7500 5 2 3.0	∞ 3.0			Died 4th day Autopsy: Pneumonia RML, 1/2 RLL. IIB: Pn. III and Gram-neg. bacillus
6.5	1325	1 5	l.t.	8-9-32	Bl. cult. X-ray WBC 14 7	∞ 1/2 RLL 7 3					Died 2nd day Autopsy: Pneumonia 1/2 RLL. IIB: Pn. III and Gram-neg. bacillus
6.8	2550	1 5	l.b.	9-6-32	Bl. cult. X-ray WBC 13 8	∞ RLL 3.6					Died 1st day Autopsy: Pneumonia 1/2 RLL.
7.3	1800	0.5	l.b.	11-8-32	Bl. cult. X-ray WBC 27 1	2240 RLL (mottled) 4.1	∞ 0.5				Died 2nd day Autopsy: Empyema. Pneumonia RLL; collapsed RML, congested RUL.
7.4	1650	0 35	l.b.	11-11-32	Bl. cult. X-ray WBC 20.7	∞ RLL RML 10.3					Died 2nd day Autopsy: Pneumonia 2/3 RLL, 2/3 RML, patchy engorgement RUL.
1.09	1400	0 3	l.b.	1-21-33	Bl. cult. X-ray WBC 21.0	2200 1/2 RUL 1/3 RML 29.6	1600 3/1 RUL 1/2 RML 19.6	+ RUL RML 9.3	.4000 Same 8.5	+ Spread 11.4	Died 6th day Autopsy: Fibrinopurulent pleurisy right; pericarditis; pneumonia RUL, RML; congestion RLL.

of the fatal cases with mild (1–250 colonies per cc.) septicemia, three or more lobes were involved.   But with the higher degrees of septicemia, death frequently occurred when only one or two lobes were involved.   In the latter animals, death occurred comparatively early in the disease, before sufficient time had elapsed for further spread to occur.   The details of the disease in different groups of animals are given below.

Group A. Lobar Pneumonia without Septicemia

In this group are included 20 monkeys, all of which recovered.

The weights varied from 950 gm. to 2200 gm., with an average of 1850 gm. The amount of culture varied from 0.1 cc. to 1.0 cc., with an average dose of 0.42 cc.   In 15 animals, only one, or part of one lobe, was involved.   The average time of recovery was 4.3 days after infection.   In those with only one lobe involved, recovery took place on an average of 3.4 days after infection, while in the others the duration of the disease averaged 6.4 days.   Among the latter, there were 2 cases in which relapses occurred, but with ultimate recovery on the 8th and 12th days respectively.   In the entire group, there was a tendency, as shown by roentgenograms, for a lesion which was well localized on the 1st day, to extend on the 2nd day, and to show evidence of beginning resolution on the 3rd day. The white blood cell count tended to increase on the day after infection, and to remain at a relatively high level throughout the course of the disease.

The charts and protocols of cases illustrative of this group are presented.

Monkey 1-16 (Chart 2) represents an example of pneumonia without septicemia, in which the disease was of brief duration.   The temperature fell by crisis on the 2nd day after infection, and resolution was evident in the roentgenogram taken on the 3rd day.

Monkey 8-9 (Chart 3) is another instance of pneumonia without septicemia. The pulmonary involvement spread through the right upper lobe and right middle lobe during the first 4 days, after which resolution began, and recovery apparently took place on the 6th day.   The following day the temperature rose again and a new pneumonic process was noted in the right lower lobe.   Extension occurred during the next 3 days, although there was no demonstrable septicemia, and leukocytosis persisted.   Recovery finally occurred on the 12th day.

M. cynomolgos No. 1-16 ♀ Weight 1850 gm. Route i.b.

	1933 May 15				16			17			18			19		
Day of disease					1			2			3			4		
Hour	9	1	2	4	5	9	1	5	9	1	5	9	1	5	9	1	5

Temperature °F. (106–97)

Blood cult. plate / broth:

| | plate | | | | | 0 | | | 0 | 0 | | | 0 | | |
| | broth | | | | | 0 | | | 0 | 0 | | | 0 | | |

W.B.C. (thousands): 21.1 ... 40.6 ... 31 ... 18.1 ... 13.7

X-ray: Normal | Major part R.M.L. | Entire R.M.L. | Rapid clearing | Further marked clearing

Morphine ¼ gr. *Pneum. I.V. 0.1 cc.* *healed well*
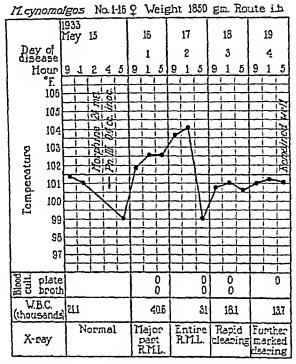
CHART 2. Experimental pneumonia showing an abortive course.

Lobar Pneumonia with Septicemia

Since the height of the septicemia accompanying experimental pneumonia in monkeys appears to bear a relation to the outcome of the disease, the remainder of the cases have been subdivided on the basis of the number of pneumococci in the circulating blood during the first 3 days after infection: 1 to 250 colonies per 1 cc. of blood—mild to moderate septicemia; 250 to 2000 colonies per cc.—heavy septicemia; 2000 colonies or more per cc.—extreme septicemia.

Group B. Lobar Pneumonia with Septicemia
(1–250 Colonies per Cc.)

This group comprises 20 monkeys. Of these, 11 recovered and 9 died, a mortality of 45 per cent.
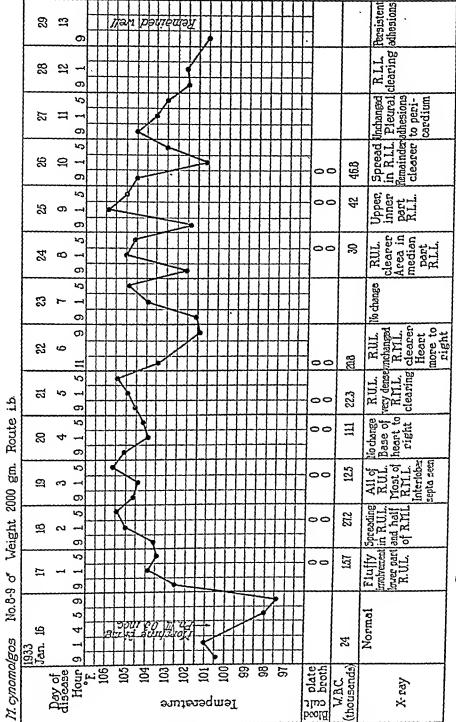
CHART 3. Experimental pneumonia with a relapse and prolonged course.

There is one case in which enumeration of colonies was not made, but which is included because the course of the disease is typical of that occurring in this group, and another case in which, although no estimates of the height of septicemia were made during life, the blood culture made post mortem contained 140 colonies of pneumococci per 1 cc. of blood. The average weight of the animals of this series was 1923 gm., the average dose of pneumococci employed was 0.68 cc., while the range of dosage in both the recovered and fatal cases was from 0.3 cc. to 1.0 cc.

Of the 11 recovered cases, only 3 had involvement of two lobes, while in the others there was only one lobe or part of one lobe involved. The average duration of the disease was 4.5 days. There was a tendency for the lesion to spread during the first 3 days. In recovered cases, the white blood count generally reached its lowest level at the time when the number of bacteria in the blood and the extent of the pulmonary involvement were greatest. A subsequent rise of the white blood cell count occurred during recovery.

In one of the 9 fatal cases, there was involvement of two lobes, in the other 8 fatal cases of three or more lobes. The average time of death was 5.4 days after infection. In 4 of these cases, Pneumococcus III was obtained from the pericardial fluid at autopsy, and in 3 empyema was found. In the 4 instances in which white blood counts are available, there was a decrease in the number of circulating leukocytes as the pneumonic process spread.

The charts of typical cases of this group are shown.

Monkey 4-5 (Chart 4) represents a case of pneumonia which recovered by crisis on the 5th day of the disease; a mild septicemia was present from the 2nd to 4th days. No depression of the leukocyte counts occurred.

Monkey 9-7 (Chart 5) serves as an example of pneumonia with mild septicemia from which the animal recovered spontaneously on the 6th to 7th day. Resolution of the pneumonic process had commenced before the septicemia had completely subsided. The leukocytes were increased in number throughout the course of the disease.

Monkey 1-6 (Chart 6) represents the type of case in which a progressively spreading pneumonia with a relatively mild septicemia terminated fatally on the 5th day. The animal showed only a slight febrile reaction. Autopsy revealed the presence of consolidation of the entire right lower lobe, two-thirds of the right middle and right upper lobes, and irregular consolidation of two-thirds of the left lower lobe.

Monkey 9-0 (Chart 7) illustrates the course of the disease in an animal in which the persistently spreading pneumonia is accompanied by a moderately severe septicemia increasing to a terminal, heavy septicemia. There was only moderate depression of the circulating leukocytes. Death resulted on the 8th day. On the day of death, a line suggesting pleural effusion was noted in the X-ray. Consolidation of the entire right lower and middle lobes and of one-half the right

M.cynomolgos No. 4-5 ♀ Weight 2050 gm. Route i.b.

Day of disease / Hour	1932 Sept. 22 (9 1 4 5)	23 — 1 (9 1 5)	24 — 2 (9 1 5)	25 — 3 (9 1 5)	26 — 4 (9 1 5)	27 — 5 (9 1 5)	28 — 6 (9 1)
Blood cult. plate		0	23	6	5	0	
Blood cult. broth			+	+	+	0	
W.B.C. (thousands)	20.4	52.8	21.3	21	25	23	
X-ray	Normal	Middle of R.L.L.	Most of R.L.L.		Denser	Unchanged	Clearing

(Chart notation: "Morphine 16 mg." and "Pn.III 0.5 cc. inoc." at Sept. 22; "Remained well" at Sept. 28.)
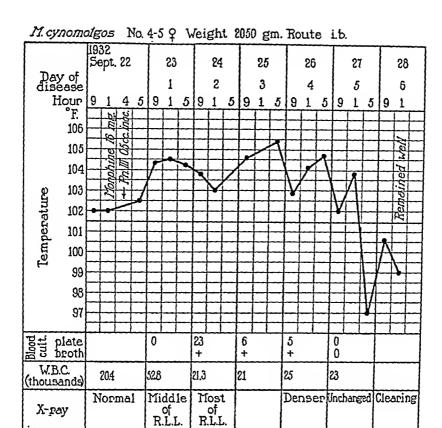
CHART 4. Experimental pneumonia with a mild septicemia followed by crisis.

M.cynomolgos No. 9-7 ♀ Weight 2700 gm. Route i.b.

Day of disease / Hour	1933 Feb. 20 (9 1 5)	21 — 1 (9 1 5)	22 — 2 (9 1 7)	23 — 3 (9 1 5)	24 — 4 (9 1 5)	25 — 5 (8 5)	26 — 6 (12 7)	27 — 7 (9 1)
Blood cult. plate		0	1	2	10	4	1	0
Blood cult. broth		+	+	+	+	0		0
W.B.C. (thousands)	26.2	47	45	30.9	28	17.8	25.6	22.6
X-ray	Normal	Lower ½ R.U.L.	Entire R.U.L. Early RML	Spreading R.M.L.	Unchanged	Clearing R.M.L.		Advanced resolution

(Chart notation: "Morphine 12 mg." and "Pn.III 0.33 cc. inoc." at Feb. 20; "Remained well" at Feb. 27.)

CHART 5. Experimental pneumonia with a mild septicemia followed by recovery.

M. cynomolgos No. 1-6 ♂ Weight 1275 gm. Route i.t.

	1932 Jan. 20	21	22	23	24	25
Day of disease		1	2	3	4	5
Hour	9 1 2	9 1 5	9 1 5	9 1 5	10	9 11 30

(Temperature chart, °F. 97–106)

Blood cult.	plate		6	133	38	37	
	broth		+	+	+	+	
W.B.C. (thousands)							
X-ray		Normal	Median ½ R.L.L.	Spreading in R.L.L. Early lesion in R.M.L.	Entire R.L.L. Hazy shadow R.L.L. & R.U.L.	Density greater on rt. L.L.L.(?)	Entire rt. lung exc. apex L.L.L.

CHART 6. Experimental pneumonia with a relatively mild septicemia terminating fatally.

M. cynomolgos No. 9-0 ♂ Weight 1950 gm. Route i.b.

	1933 Jan. 23	24	25	26	27	28	29	30	31
Day of disease		1	2	3	4	5	6	7	8
Hour	10 1 5 10	9 1 5	9 1 5	9 1 5	9 1 5	9 12 4	11 10	9 1 5	9

(Temperature chart, °F. 97–106)

Blood cult.	plate		33	32	235	174	155	1000	8	
	broth		+	+	+	+	+	+	+	
W.B.C. (thousands)	232	13.5	27	25	11.1	18		10	
X-ray	Normal	Lateral ⅔ R.L.L.	Most of R.L.L. R.M.L.?	Enlarged	Spread in R.M.L.	Spreading		Spreading in R.M.L.	Spreading Pleural line
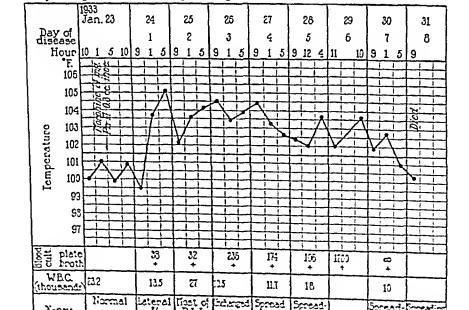
CHART 7. Experimental pneumonia showing a persistent spread with a moderately severe septicemia followed by a terminal heavy septicemia.

upper lobe was found at autopsy.   Fibrinopurulent pleurisy and pericarditis were present.

Group C. Lobar Pneumonia with Septicemia
(250–2000 Colonies per Cc.)

The animals included in this group present a more severe form of the disease than those of the preceding group.   Of 12 animals, 3 recovered, a mortality of 75 per cent.

The height of the septicemia in the 3 recovered animals during the first 3 days was 700, 468, 366 colonies per 1 cc. of blood, respectively, while in the fatal cases the highest was 1812 colonies per 1 cc.   The average duration of the disease in the recovered cases was 6 days, in the fatal 4.6 days.   The average weight of the recovered animals was 2120 gm.; that of the animals which died was 2280 gm. The inoculum of Type III Pneumococcus in recovered animals was 0.2, 2.0, 0.5 cc., respectively, with an average of 0.9 cc.; in fatal cases the average was 0.6 cc., with a range from 0.05 cc. to 1.0 cc.   In 2 of the recovered cases only one lobe was involved, in the other, two lobes.   In 2 of the fatal cases one lobe was involved, 2 had two lobes affected, and in 5 there was involvement of three or more lobes. In 6 of the 9 fatal cases, Pneumococcus III was obtained from pericardial or pleural fluid at autopsy.

Charts illustrating the variations in the course of infection in this group are presented.

Monkey 1-27 (Chart 8) is an instance in which recovery occurred following a severe form of the disease.   The pneumonia spread during the first 3 days, with the septicemia reaching its height (366 colonies per cc.) at that time.   On the 4th day resolution began, and the blood was sterile on the 5th day.   The leukocyte count varied inversely with the septicemia.

Monkey 6-1 (Chart 9) represents the type of case in which a progressively spreading lesion with increasing septicemia terminated fatally on the 5th day. The white blood count fell steadily.   At autopsy, complete consolidation of the right lower and middle lobes and of the left middle lobe was found.

Monkey 9-6 (Chart 10) is an example of the course of the disease in an animal in which, with a spreading pneumonic lesion, septicemia is absent at first, then mounts rapidly to a fatal outcome on the 5th day.   The leukocytes were maintained at a good level throughout.   A pneumothorax, apparently spontaneous, occurred on the uninvolved side on the 4th day.   The chart also records the depressant effect of a preliminary injection of morphine on the temperature of the animal.   Autopsy revealed a left-sided pneumothorax with partial collapse of the lung.   On the right side, consolidation was complete in the upper and middle lobes

	1933 June 5	6	7	8	9	10
Day of disease		1	2	3	4	5
Hour	9 1 5	9 1 5	9 1 5	9 1 5	9 1 5	9 12

Blood cult.	plate broth		140	35	365 +	2 0	0
W.B.C. (thousands)	21	40	14.1		13.7	6.8	3.4
X-ray	Normal	Lower ⅔ R.U.L.	Entire R.U.L. Early RML	Spread in RML.	R.U.L. clearing	Enlarged
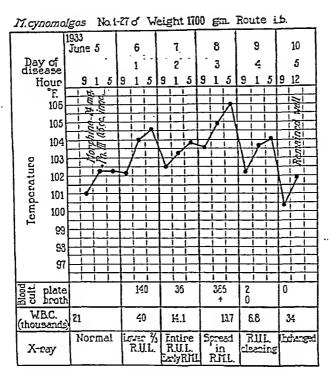
CHART 8. Experimental pneumonia with a moderately severe septicemia followed by recovery.

	1932 Oct. 4	5	6	7	8	9
Day of disease		1	2	3	4	5
Hour	2 3 5	9 1 5	9 1 5	9 1 5	9 1 5	10

Blood cult.	plate broth		35	24	440 +	440	0
W.B.C. (thousands)	11.2	2.4	15		14.5	2.5	8.3
X-ray	Normal	Upper outer part R.L.L.	Most of R.L.L.	R.L.R.L. Early in LML	More in L.M.L.	
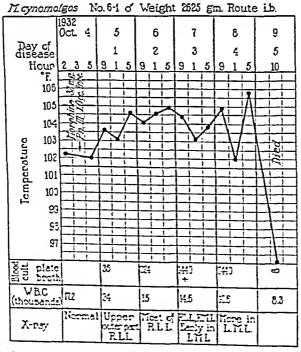
CHART 9. Monkey 6-1 represents the type of case with a spreading lesion and increasing septicemia terminating fatally.

M. cynomolgos No.9-6 ♂ Weight 2600 gm. Route i.b.

	1933 Feb. 15	16	20	21	22	23	24	25
Day of disease	A	B	C	1	2	3	4	5
Hour	9 1 5	9 1 5	9 1 5	9 1 5	9 1 7	9 1 5	9 1 5	9

(Temperature chart, °F, range 97–106. Notations: "Morphine 24 mg." under Feb. 15; "Morphine 24 mg. Pn.III 0.33 cc. i.b." under Feb. 20; "Found dead" under Feb. 25.)

Blood cult.								
plate				0	10	1220	∞	
broth				0	+	+	+	

| W.B.C. (thousands) | 31 | | 18.2 | 22.3 | 20.2 | 23.6 | 27.8 | |

| X-ray | Normal | | | Lower part R.U.L. | Spreading | Entire R.U.L. Early in R.M.L. | Spread Pneumothorax left | |
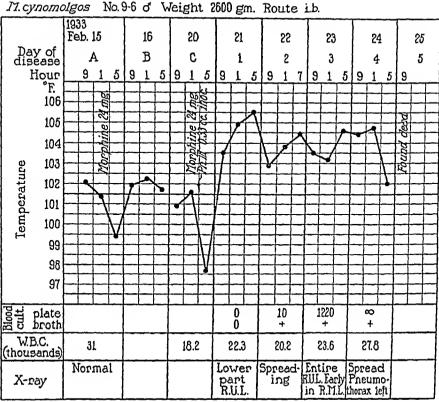
CHART 10. Experimental pneumonia with negative blood culture at first, followed by a rapidly mounting septicemia. Note depressant effect of preliminary injection of morphine on temperature.

and present in one-third of the lower lobe. Fibrinopurulent pleurisy was also present. Roentgenograms taken during the course of the disease are shown in Figs. 7 to 11.

Group D. Lobar Pneumonia with Septicemia
(More than 2000 Colonies per Cc.)

In this group of 16 monkeys, there were no recoveries. The infection in these animals was characterized by the frequent early involvement of more than one lobe, sometimes diffuse, a rapidly mounting septicemia, exhaustion of leukocyte response, and early death.

The average weight of the animals was 1670 gm.; the average infecting dose was 0.79 cc., with a maximum of 1.5 cc. and a minimum of 0.3 cc. In 2 monkeys, only one lobe was involved; in 6, two lobes were affected, while in the remaining 9 animals three or more lobes were involved. In 6 instances, empyema, peri-

carditis, or both, were present. The average duration of the disease was 2.8 days. In several instances at autopsy, in addition to Type III Pneumococcus, Gram-negative bacteria of the *B. coli* or *B. lactis aerogenes* group were found. These organisms were considered terminal invaders. It is of interest to note that in several instances they were present in the throat cultures of the same animals before infection.

M. cynomolgas   No. 2-6 ♀   Weight 1750 gm.   Route i.t.

	1932 Feb. 29	Mar. 1	2	3	4	5
Day of disease		1	2	3	4	5
Hour	9 1 245 5	9 1 5	9 1 5	9 1 5	9 1 5	9
Blood cult. plate broth		340 +	1600 +	5280	2260	
W.B.C. (thousands)		15	10	7.2	6.1	
X-ray	Normal	Inner ½ R.L.L. & R.M.L.	R.L.L.R.M.L. Same in R.U.L.	Entire R.L.L. R.M.L. & mod. in R.U.L.	Increased density	Entire rt. lung. Inner part LLL
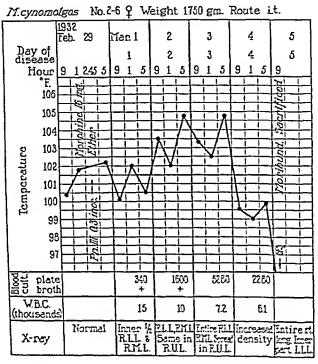
CHART 11. Experimental pneumonia with severe septicemia the first 3 days of disease.

The charts of representative cases are presented.

Monkey 2-6 (Chart 11) is an example of a consistently spreading pneumonia with a septicemia of 5280 colonies per 1 cc. of blood on the 3rd day. There was a progressive depression of the leukocytes. The animal was sacrificed when moribund on the 5th day. Consolidation of the entire right lung, as well as of the lower part of the left lower lobe, was found.

Monkey 7-3 (Chart 12) represents the rapidly fatal type of disease with empyema and extreme septicemia. In this instance there was almost a complete

exhaustion of the leukocytes on the 2nd day, when death occurred. The temperature was subnormal throughout. At autopsy, 6 cc. of seropurulent fluid was found in the right pleural cavity. The entire right lower lobe was consolidated; the right middle lobe was collapsed and covered with gelatinous exudate; the right upper lobe was markedly congested.

CHART 12. Experimental pneumonia representing the rapidly fatal type of disease with empyema and extreme septicemia.

DISCUSSION

In the present group of 68 monkeys, in which intrapulmonary infection was established by the intratracheal or intrabronchial inoculation of Type III Pneumococcus, a disease closely resembling lobar pneumonia in man was produced. In 50 per cent of the animals, the disease terminated fatally. There was usually an initial pneumonic consolidation of one lobe, or part of one lobe, which then spread to involve a greater pulmonary area. An initial rise of the circulating white blood cells was commonly followed by a decrease at the height

of the disease. The temperature was elevated during the disease, and usually fell with a critical drop at the time of recovery. Invasion of the blood by Type III Pneumococcus occurred in 70 per cent of the cases.

The severity of the disease varied with individual animals. Of 20 monkeys in which pneumonia was unaccompanied by septicemia, all recovered, many of them after a brief course during which comparatively little extension of the primary lesion occurred. On the other hand, no recoveries occurred in 16 monkeys in which the pulmonary infection was associated with extreme septicemia. In these instances, subnormal temperature, marked depression of the leukocytes, and rapidly mounting septicemia were followed by early death; at times there was diffuse pulmonary involvement. Empyema and pericarditis were noted frequently.

In the intermediate groups in which pneumonia was accompanied by moderate or heavy septicemia, the disease ran a more uniform course, with a duration of 4.5 to 6 days. In animals in which the septicemia was moderate (1–250) during the first 3 days, the mortality rate was 45 per cent; with heavy septicemia (250–2000) accompanying pneumonia, the mortality rate reached 75 per cent.

Although a number of factors are involved, the degree of septicemia accompanying the pneumonia appears to be the most useful clinical index of the ultimate outcome of the disease in the individual case. With septicemia, more progressive extension of pneumonia is observed, the depression of leukocytic response is more marked, and the general illness of the animal is usually more pronounced. In contrast to Type III pneumococcus pneumonia in man, the septicemia which accompanies experimental lobar pneumonia in monkeys is of a higher order, and spontaneous recovery may occur after a degree of septicemia rarely encountered even in fatal cases in man. Nevertheless, in the marked inconstancy of the clinical picture, experimental pneumonia of the monkey resembles Type III pneumococcus pneumonia in the human being.

Under the conditions of the present study, it has been impossible to predict in advance the probable course of experimentally induced pneumonia in the individual monkey. The summary of experience has been that the greatest variable is the individual resistance of the

animal, which is probably the resultant of many interrelated factors. Attempts were made, therefore, to analyze the different measurable variables and, if possible, to correlate them with the differences observed in the course and outcome of the disease in different animals.

The virulence of the particular strain of Type III Pneumococcus used throughout these experiments was, so far as could be determined, relatively constant. This is shown by the fact that when tested in rabbits, after an interval of 9 months, its original virulence for that species was unchanged.

In a number of instances, studies were made of the pneumococcidal power of the blood of monkeys before infection, but the results were uniformly negative. Similarly, skin tests with pneumococcus nucleoprotein revealed no suggestion of previous sensitization, since the tests elicited no reaction in the normal animals. These observations afforded no evidence of preceding immunization such as has been emphasized by Wadsworth (9) and Stillman (10).

A survey of the original white bood counts, the counts done on the 1st day after infection or averaged during the first 3 days of the disease, presented no evident relationship between the height of the early counts and the course or outcome of the pneumonia. There was, nevertheless, a distinct tendency for the number of circulating leukocytes to fall during the height of the disease and to rise with recovery. Less constant was the tendency for the leukocytes to rise when empyema or pericarditis occurred, even though these cases always terminated fatally. A similar lack of correlation between the white blood counts and the outcome of experimental pneumonia was also observed by Blake and Cecil (1) in their study.

As regards the influence of the size of the infecting dose of organisms upon the character of the resulting disease, it may be stated that the mortality rate was higher in the animals which received the largest doses of culture. There was, however, marked variation in the severity of the disease in individual animals regardless of the amount of culture inoculated. Furthermore, the extent of pulmonary involvement and the height of the septicemia following infection with the large doses of organisms was no greater than that frequently seen in animals receiving the small doses. The same facts are demonstrated when the dose is computed on the basis of body weight.

There appears, therefore, to be no clear relationship between the size of the infecting dose and the outcome of the experimental pneumonia.

General atmospheric conditions apparently play a rôle in the resistance of the animals to infection. In general, during the winter the type of infection produced with small doses of organisms was similar to that obtained in the warm weather with larger doses of organisms. The individual variation was noteworthy at all times, however.

The severity of the depressant action of ether or morphine, indicated by stupor and subnormal depression of body temperature, differed considerably in different animals. There was apparently a tendency for monkeys in which the depressant effect was most marked to be the sicker. Furthermore, the inhalation of ether is known at times to produce in experimental animals a hemorrhagic edema of the lungs. Temporary lowering of the physiological resistance of an experimental animal, so as to allow bacteria introduced into the respiratory tract to gain a foothold, has been described following chilling, alcohol, morphine, fatigue, and deficient diets. In the present study, it is not unlikely that differences in the effect of morphine or ether upon different animals may have had a definite influence upon the severity of the disease in these animals.

Localization of the infecting material should undoubtedly have a distinct influence upon the course of the disease. In the great majority of animals which recovered spontaneously, only one or two lobes were involved, whereas with more widespread involvement a high percentage of fatalities occurred (Chart 1). With the intratracheal method, in which ether anesthesia was used, there is a somewhat greater tendency for the infecting material to be distributed more widely than when the intrabronchial method is employed. Reference to Table II, however, reveals similar forms of disease produced by both methods. With the intratracheal route, the early consolidation was most frequently noted by X-ray in the median portion of the involved lobe, whence it spread to involve the entire lobe; with the intrabronchial method, by which the infecting material is placed farther out in the bronchial tree, the original consolidation was first seen in a more lateral position in the lobe, spreading medially. In either case, the lesion may progress to involve one or more lobes completely, or the process may be limited to part of a lobe.

The use of starch as a protective medium for the bacteria, as suggested by Terrell, Robertson, and Coggeshall (8), has been found to be unnecessary for the production of lobar pneumonia in the Java monkey. In 8 animals inoculated by the intrabronchial route with broth cultures of Type III Pneumococcus alone, the results were quite similar to those obtained when starch was employed. In these instances, again, distinct variations in the course of the disease in individual monkeys was noted.

The features mentioned are those which are considered to play a rôle in influencing the course of the experimental pneumonia due to Type III Pneumococcus after it has been established. A discussion of the pathogenesis and pathology will appear in a subsequent communication.

The results of the present study furnish evidence that, with Type III Pneumococcus, pneumonia of lobar distribution comparable in its clinical features to human lobar pneumonia can be produced in monkeys. The course of the disease is variable, and under existing conditions the result in individual animals is not predictable in the early stages of the disease.

SUMMARY

It has been possible by the intratracheal or intrabronchial inoculation of Type III Pneumococcus to produce in monkeys of the *M. cynomolgos* species an experimental pneumonia which in its clinical aspects closely resembles pneumococcus lobar pneumonia in man. The experimental disease is characterized by the development of a well localized pulmonary lesion of lobar distribution which tends to spread, the frequent occurrence of septicemia, a sustained fever, and the termination of the infection after a variable interval, in recovery or death of the animal. Wide variations in the severity of the disease in different monkeys have been noted. These variations appear to be due primarily to differences in the resistance of individual animals. The height of the septicemia accompanying the experimental pneumonia has been found to be the most valuable objective index of the probable outcome of the disease. Other factors which may influence the course and outcome of the disease are discussed.

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EXPLANATION OF PLATES

PLATE 45

The intrabronchial method of inoculation demonstrated by the injection of lipiodol.

FIGS. 1 to 3. Roentgenograms of monkey's lung showing catheter in upper bronchus of right lower lobe and the different stages in the injection of 0.5 cc. of lipiodol.

FIG. 4. Lateral view of same animal.

FIG. 5. Roentgenogram of monkey's chest after the injection of 0.5 cc. of lipiodol into both the left lower lobe and the lower part of the right upper lobe.

FIG. 6. Same as Fig. 5, with animal in lateral position.

PLATE 46

Roentgenograms taken during the course of experimental pneumonia in Monkey 9-6, which terminated fatally.

FIG. 7. Control. Before infection (Feb. 10).

FIG. 8. 1st day after infection (Feb. 21), showing early consolidation in the right upper lobe.

FIG. 9. 2nd day (Feb. 22), showing spread of pneumonia in the upper part of the right upper lobe.

FIG. 10. 3rd day (Feb. 23), showing complete involvement of the right upper lobe and probably extension into the right middle lobe.

FIG. 11. 4th day (Feb. 24), showing a left pneumothorax together with a well marked involvement of the right upper and middle lobes (Chart 10).
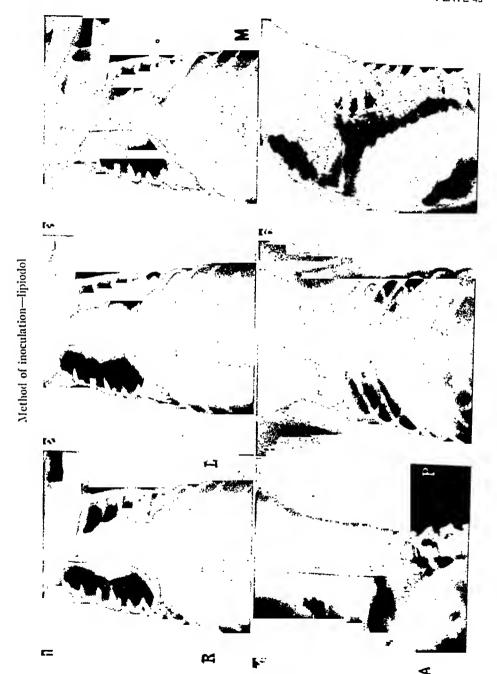
PLATE 47

Roentgenograms of fatal pneumonia in Monkey 5-2.

FIG. 12. Control. Before infection (June 8).
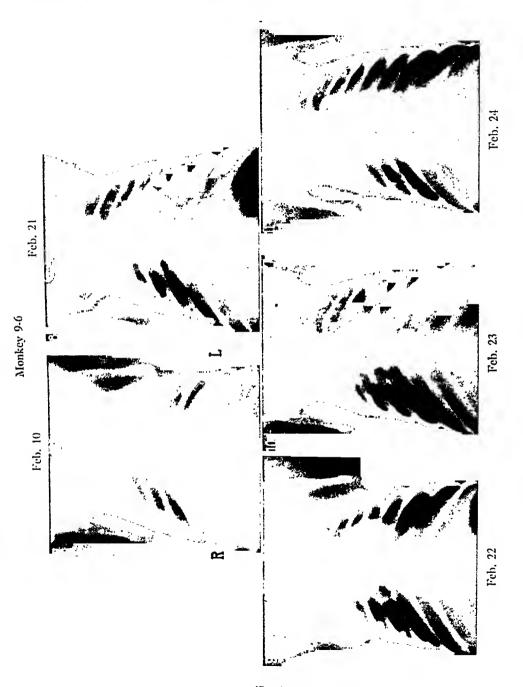
FIG. 13. 1st day after infection (June 10), showing early pneumonia in right middle lobe.

FIG. 14. 4th day (June 13), showing a more diffuse pneumonia involving most of the right middle and upper lobes, the median part of the right lower lobe, and the lower part of the left upper lobe.

PLATE 45

Method of inoculation—lipiodol
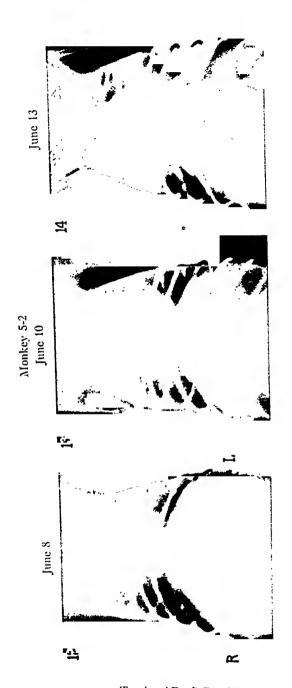
(Francis and Terrell: Type III pneumococcus pneumonia. I)

(Francis and Terrell: Type III pneumococcus pneumonia. I)

EXPERIMENTAL TYPE III PNEUMOCOCCUS PNEUMONIA IN MONKEYS

II. Treatment with an Enzyme Which Decomposes the Specific Capsular Polysaccharide of Pneumococcus Type III

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PLATES 48 TO 50

In the preceding paper (1) the production and the clinical features of experimental Type III pneumococcus pneumonia in monkeys of the *M. cynomolgos* species were described. The experimental disease in its clinical aspects closely resembles lobar pneumonia in man. The infection in these animals results in a pneumonic lesion of lobar distribution which tends to spread and which terminates after a variable period in spontaneous recovery or death. The irregularity of the course of the disease in individual animals is striking, but when septicemia is present the mortality rate increases proportionately with the number of organisms in the circulating blood. Thus, the height of the septicemia, irrespective of the size of the infecting dose, was found to be the most valuable objective index of the severity of the disease in a given animal.

In earlier studies, Avery and Dubos (2) showed that a specific enzyme of bacterial origin was capable of protecting mice against subsequent infection with Type III Pneumococcus, and of exerting a curative effect on infections already established. The beneficial effect of the enzyme was demonstrated to be due to its capacity to decompose the specific capsular polysaccharide of Type III Pneumococcus, thus rendering the bacteria readily susceptible to phagocytosis by the cells of the animal body.

Studying the effect of the enzyme upon a dermal infection with Type III Pneumococcus in rabbits, Goodner, Dubos, and Avery (3)

found that 95 per cent of untreated rabbits died, while of those which were treated with enzyme, 95 per cent recovered. With the amounts of enzyme employed, there was found to be a degree of infection which, although influenced by enzyme, terminated fatally. Goodner and Dubos (4) later showed that the amount of enzyme required for successful therapy increased with the height of the septicemia.

Since it had been found possible regularly to produce an experimental pneumonia in monkeys very similar to lobar pneumonia in man, it was of interest to determine the effect of treatment with specific enzyme upon the course and outcome of the disease. It was fully recognized that the methods employed in the production of enzyme were not standardized, and that different lots of enzyme varied considerably in their potency. Certain lots even contained substances which were toxic for animals. In spite of these technical imperfections, the treatment of experimental lobar pneumonia in monkeys was begun. The present paper reports the results of enzyme treatment of experimental Type III pneumococcus pneumonia in 40 monkeys. All animals were treated within the first 3 days after infection. Consequently, they have been divided into groups on the basis of the height of the septicemia during the first 3 days of the disease. This classification affords a basis for comparison of the results with the 68 untreated monkeys included in the preceding paper.

EXPERIMENTAL

Selection of Animals for Treatment.—Because of the extreme variations in the severity of the experimental disease in different monkeys, it was not feasible to select alternate animals for treatment and controls. Only after the disease was established was it possible to determine which animals in a given experiment were the most likely to succumb. Consequently, the sickest animals in each experimental group (2 to 6 in number) were chosen for enzyme therapy. The criteria on which the choice was based were the fever, the number of circulating leukocytes, the X-ray evidence, the general condition of the animal, and especially the height of the septicemia.

Enzyme.—The preparations of the enzyme were made by methods previously described (5); but from time to time minor changes were introduced in attempts to produce a more potent product. The enzyme content of the different preparations varied from 2 to 20 units per cc.

Procedure of Treatment.—Practically all therapeutic experiments were carried out during the winter and spring, when experimental conditions were more nearly

stable and the smaller doses of organisms were more efficient in producing typical pneumonia. During the period of treatment the animal's temperature was recorded, repeated blood cultures and blood counts were made, and the X-rays were carefully studied for evidence of extension or regression of the pneumonic process. A blood culture and white blood cell count were made immediately before treatment. Because of variation in the enzyme content of different lots, the usual procedure was to administer 10 cc. of the enzyme preparation intravenously. Further treatment was based upon the response to the first injection, the severity of the disease, and the potency of the particular preparation of enzyme. Additional treatments were given as often as three times daily, either intravenously, intraperitoneally, or by both routes simultaneously. When enzyme therapy was employed, treatment was always begun within the first 3 days after infection.

TABLE I

*Mortality in Experimental Type III Pneumococcus Pneumonia in Monkeys Receiving Enzyme Therapy**

Diagnosis	No. of animals	No. recovered	No. died	Mortality
				per cent
Pneumonia without septicemia.............	8	8	0	0
Pneumonia with septicemia (1–250 per cc.)..	15	15	0	0
Pneumonia with septicemia (250–2000 per cc.)...................................	9	8	1	11.1
Pneumonia with septicemia (2000 or greater per cc.)................................	8	1	7	87.5
Total......................................	40	32	8	20.0
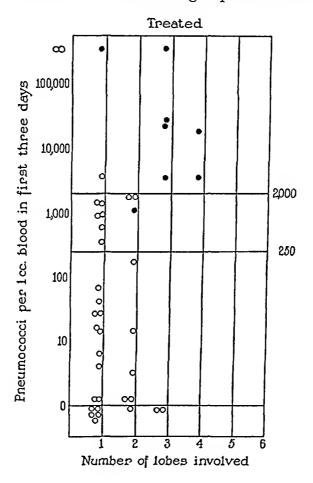
* Classified on the basis of height of septicemia during first 3 days.

No attempt was made to ascertain the minimal amount of enzyme required in individual monkeys, but, rather, treatment was intensively employed until the result was assured.

RESULTS

In the entire series of 40 monkeys with pneumonia, in which enzyme therapy was employed, the mortality rate was 20 per cent. The animals have been subdivided into four groups based on the height of the septicemia during the first 3 days of the disease (Table I). The distribution of the cases is comparable to that of the untreated series (1). With one exception, the fatal cases fall into the group with extreme septicemia and marked prostration. The height of the septi-

cemia during the first 3 days, and the number of pulmonary lobes involved, in relation to recovery or death, are represented graphically in Chart 1.   The amount of pulmonary involvement was, in general, greater in the fatal cases than in those which recovered.   The salient features of the disease in the different groups are discussed in detail.

CHART 1. The relation of the height of septicemia and the amount of pulmonary involvement to the outcome of the disease.

Group A. Lobar Pneumonia without Septicemia

In this group of 8 animals, no demonstrable invasion of the blood had occurred at the time treatment was begun, which in 6 instances

was on the 1st day, in 2 on the 2nd day after infection. All 8 animals recovered. The average weight of the group was 2130 gm. The average amount of culture employed for inoculation was 0.35 cc. In 2 cases there was involvement of three pulmonary lobes, in 1 case two lobes, while in the remaining 5 only one lobe was involved. The average duration of the disease was 3.2 days after infection. This was estimated, to a great extent, upon the time when regression of the pneumonia was noted in the X-ray. The temperature curve and the white blood count were of less value, since some febrile reaction and fall in the number of circulating leukocytes frequently followed administration of enzyme. When a rise in the number of leukocytes began, however, recovery was usually definitely established.

No attempt was made to ascertain the minimal amount of enzyme required. The number of units of enzyme given to animals in this group varied from 100 units to 360, and the number of treatments varied from a single dose to four over a period of 3 days. In most instances, improvement began shortly after treatment was instituted. The monkey became more alert; there was a tendency for the fever to subside; regression of the shadow, most noticeable at the margins, was observed in the X-ray film.

In comparison with the untreated animals (1) without septicemia, the course of the disease in the treated animals was, on the average, of shorter duration. Furthermore, from a clinical standpoint, the animals of this group were, at the time treatment was begun, sicker than those of the untreated group, and the amount of pulmonary involvement was greater. In some instances, it is possible that with a spreading pneumonia, septicemia and death might have occurred in the absence of treatment. In no instance was a prolonged illness noted, although one monkey, No. 4-2, while recovering from pneumonia, developed cutaneous sores and on the 6th day a septicemia due to *Staphylococcus aureus* and a Gram-negative bacillus. When sacrificed, the lungs showed definite resolution and the cultures from the pneumonic lobes revealed no pneumococci.

Monkey 1-11 (Chart 2) was treated on the 2nd day after infection, when the right upper lobe was completely involved. At that time the temperature was high, but following a single treatment with 50 units of enzyme there was a critical fall of the fever; no spread of the lesion occurred. On the 3rd day another treat-

TABLE II

Experimental Type III Pneumococcus Pneumonia in Monkeys Receiving Enzyme Therapy

No.	Weight	Dose	Route*	Date		Days after infection — Pneumonia without septicemia							Result†	Remarks
	gm.	*cc.*				1	2	3	4	5	6	7		
3-3	1650	0.4	i.t.	4-11-32	Bl. cult.‡	0 0	0	0	0	0			R 2	
					WBC 17.7§	12.6, 8.1	6.9	8.1	16.6	20.4				
					X-ray‖	1/3 RUL 1/3 RML 1/3 RLL	Clearing	Clearing	Clearing	Clearing				
					Enzyme units	49								
4-0	2150	0.4	i.t.	5-10-32	Bl. cult.	0 0	0	0					R 3	
					WBC 23.0	12.8	35, 35	17.5						
					X-ray	1/2 RLL 1/2 RML 1/2 RUL	Spread							
					Enzyme units	120, 120	120							
4-2	2350	0.4	i.t.	5-10-32	Bl. cult.	0 0	120	0	0				R 3	
					WBC 15.4	5.9	1.1	1.8	1.7					
					X-ray	1/2 RLL	RLL RML	RLL RML	Clearing	Clearing	Clearing			
					Enzyme units	120, 120	120							
7-6	2150	0.25	i.b.	12-12-32	Bl. cult.	0 0							R 4	Sacrificed 9th day. Many sores over body. Pneumonia RLL (resolving); RML, same; RUL, same; HB culture: Staph. and Gram-neg. bacillus. Culture: RLL negative
					WBC 21.5	24.0	12.5	8.0	3.6	5.1				
					X-ray	1/3 RLL	3/4 RLL	Clearing	Clearing	Clearing				
					Enzyme units	60	60	60						
										Staph, Gram-neg. bacillus 5.5	Staph, Gram-neg. bacillus 1.7			
7-3	2150	0.25	i.b.	12-12-32	Bl. cult.	0 0							R 3	
					WBC 18.8	23.8	10.5	20.6	14.1					
					X-ray	1/2 RLL	Same	Clearing	Clearing	Clearing				
					Enzyme units	80	30							

No.		Inoc.	Date	Determination	Successive values	R†	Remarks
1-1	2×10⁹ 25	i.b.	12-10-32	Bl. cult.	0, 0, 0	R 4	Sacrificed 5th day. RUL consolidated throughout, resolving in lower portion. Cultures: RUL negative
				WBC	17 0, 54 2, 31 4, 15 1, 11 5, 28 6		
				X-ray	1/2 RUL, Spread, RUL, Clearing, Clearing		
				Enzyme units	60, 85, 85		
1-11	10⁹ 4	i.b.	5-15-31	Bl. cult.	0, 0, 0, 0	R 4	
				WBC	18 1, 28 3, 19 2, 12 8, 12 6		
				X-ray	2/3 RUL, RUL, RUL. RML?, Clearing, Clearing		
				Enzyme units	50		
1-19	17 0 45	i.b.	5-20-33	Bl. cult.	0, 0, 0, 0	R 3	Sacrificed 16th day. Upper 1/2 RLL, yellow and flabby suggesting advanced resolution
				WBC	14 6, 20 8, 20 2, 15 1, 13 7		
				X-ray	1/3 RLL, 1/2 RLL, Clearing, Clearing, Clearing		
				Enzyme units	100, 50		

Pneumonia with septicemia, 1–250 colonies per cc. in first 3 days after infection

No.		Inoc.	Date	Determination	Successive values	R†	Remarks
2-9	1600 0 3	i.t.	3-14-32	Bl. cult.	14,0, 0, 0, 0, 0	R 3	
				WBC	20 3, 20 0, 5 2, 8 5, 15 3, 19 8, 18 9		
				X-ray	RML, 1/2 RLL, Denser, Clearing, Clearing, Clearing		
				Enzyme units	40, 20, 20		
3-0	2300 0 3	i.t.	3-14-32	Bl. cult.	30,0, 0, 0, 0, 0	R 3	
				WBC	14 8, 12 0, 4 0, 9 1, 9 0, 21 6		
				X-ray	1/2 RLL, Same, Clearing, Clearing, Clearing		
				Enzyme units	40, 20, 20		
3-4	1600 0 4	i.t.	4-14-32	Bl. cult.	0,+, 0, 0, 0	R 2	
				WBC	18 4, 12 7, 7 2, 5 6, 7 8		
				X-ray	1/2 RLL, Clearing, Clearing, Clearing		
				Enzyme units	42, 42,42, 21		

* i.t. = intratracheal inoculation; i.b. = intrabronchial inoculation.
† R = recovery. D = death. Numerals indicate the day of recovery or death. F. D. = found dead.
‡ Number of pneumococci obtained in poured plate culture per 1 cc. of blood; + = growth occurred in broth cultures of blood.
§ White blood cells in thousands per c. mm. of blood.
‖ RUL, RML, RLL, LUL, LML, LLL = right upper, right middle, right lower, left upper, left middle, left lower lobes, respectively.
(Cardiac lobe not included.)

TABLE II—*Continued*

Pneumonia with septicemia, 1–250 colonies per cc. in first 3 days after infection—*Concluded*

No.	Weight	Dose	Route*	Date		1	2	3	4	5	6	7	Result†	Remarks
	gm.	*cc.*												
6-2	2350	0.35	i.b.	11-1-32	Bl. cult.	5	2, 0	0	0				R 4	
					WBC	22.4	10.7	11.7	9.6					
					X-ray	1/2 RLL	Spread	RLL RML	Same		Clearing			
					Enzyme units		80	80						
7-7	2150	0.25	i.b.	12-12-32	Bl. cult.	+	0	0	0				R 4	
					WBC	23.8	19.2	11.6	17.3					
					X-ray	1/3 RLL	Denser	Spread	Clearing	Clearing				
					Enzyme units		60							
8-4	1950	0.25	i.b.	1-1-33	Bl. cult.	50	70	0	0	0			R 4	
					WBC	22.4	18.0	17.1	11.3	20.6				
					X-ray	1/3 RLL	1/2 RLL	Spread	Clearing	Clearing	Clearing			
					Enzyme units		200	200						
8-6	2200	0.3	i.b.	1-11-33	Bl. cult.	0	+	0	Gram-neg. bacillus 16.0	Gram-neg. bacillus 27.2			R 3	Maintained septicemia of Gram-neg. bacillus. Developed swollen, contractured legs. Died 15 days later. Lungs clear Cultures: HB Gram-neg. bacillus. RLL negative
					WBC 22.5	7.6	11.8	7.8	Clearing	Clearing				
					X-ray	RUL 1/3 RLL	RUL 1/2 RLL	Clearing 2/3 RUL	Clearing					
					Enzyme units		200							
8-8	2100	0.3	i.b.	1-16-33	Bl. cult.	3	8	0	0				R 5	
					WBC	14.6	20.0	8.5						
					X-ray	Spread	2/3 RUL	2/3 RUL	Clearing					
					Enzyme units	180	200							
9-2	2050	0.3	i.b.	1-30-33	Bl. cult.	10	6	0	0				R 4	
					WBC 19.7	8.4	6.8	8.6	21.4					
					X-ray	1/2 RLL	RLL	Clearing	Clearing					
					Enzyme units	200	180							

													Notes
2160 0 39	1 h.	7-6-33	Bl. cult.		41	13	14,18	1	4	0	0	R 7	On the 4th, 5th, and 6th days a Gram-neg. bacillus was also found in blood stream. Enzyme contaminated
			WBC 22.7		19.0	15.7	4.6	10.4	14.4	18.2			
			X-ray		2/3 RUL.	RUL.	Denser	Clearing	Clearing				
			Enzyme units			200	120,130,120	120					
1-19 1800 0 4	1 h.	5-15-33	Bl. cult.	166	60	0,0	0	Clearing				R 3	
			WBC 26.0	42.8 RML.	18.0 RML.	9.2,6	12.8						
			X-ray		1/3 RUL.	Clearing	Clearing						
			Enzyme units		50								
1-14 1800 0 4	1 h.	5-15-33	Bl. cult.	11	0	50,50	0					R 3	Compare with No. 1-1, preceding paper
			WBC 13.9	30.5 RML.	13.6 Same	11.3 Clearing	8.0 Clearing						
			X-ray		50								
			Enzyme units										
1-20 1800 0 15	1 h.	5-23-33	Bl. cult.	6	5	0	0					R 3	
			WBC 19.9	23.0 RML.	11.4 RML.	6.9 Clearing	9.7 Clearing						
			X-ray		90	90							
			Enzyme units										
1-21 1700 0 15	1 h.	5-23-33	Bl. cult.	6	43	0	0					R 4	
			WBC 21.1	25.2 RLL.	15.2 RLL.	15.0 1/2 RUL. RLL.	20.4 Clearing						
			X-ray	2/3 RLL.		90	RUL. 55						
			Enzyme units		90								
1-21 1750 0 15	1 h.	5-20-33	Bl. cult.	0	1	+	0	Clearing				R 5	
			WBC 21.1	31.0	19.1	22.6	10.2						
			X-ray	1/2 RUL.	Denser	2/3 RUL. RLL.	RUL. 55						
			Enzyme units			100							

Pneumonia with septicemia, 250-2000 colonies per cc. In first 3 days after infection

1-8 2200 0 1	1 t.	1-18-32	Bl. cult.	3	311,2	0	0		0			R 4	
			WBC 15.6	29.0 RLL.	21.0,16.0	10.3	17.1		36.3				
			X-ray	1/2 RLL.	3/1 RUL.	Denser	Clearing		Clearing				
			Enzyme units		67,15	40	15						
8-0 1900 0 25	1 h.	12-19-32	Bl. cult.	13	360	1700	400	401	6	516		R 8	Blood culture on 8th day sterile
			WBC 18.3	12.6 RLL.	8.1 RLL.	1.1 RLL.	1.0 RLL. RML.	30.2	29.0	37.0			
			X-ray	1/3 RLL.	2/3 RLL.		Clearing	Clearing	Clearing				
			Enzyme units		85	85	170			170			

TABLE II—*Continued*

No.	Weight gm.	Dose cc.	Route*	Date		1	2	3	4	5	6	7	Result†	Remarks
										Days after infection				
					Pneumonia with septicemia, 250–2000 colonies per cc. in first 3 days after infection—Concluded									
8-7	2250	0.3	i.b.	1-11-33	Bl. cult.	1	414	995	0	4	0	0	R 6	
					WBC 14.9	14.3	9.6	5.7	4.9	7.2	16.0	12.0		
					X-ray	1/2 RLL	2/3 RLL	RLL	Same	Clearing	Same	Clearing		
					Enzyme units			360	258		200			
9-5	2075	0.33	i.b.	2-6-33	Bl. cult.	536	1600	1728	141	157	15	0	R 7	
					WBC 21.0	16.2	8.5	8.7	14.6	22.5		44.0		
					X-ray	RUL	1/3 RML	Same	Clearing	Clearing				
					Enzyme units	190	200							
7-2	1275	0.33	i.b.	2-27-33	Bl. cult.	1400,4	19	174,126,120	0	0	0		R 4	
					WBC 14.7	4.2	3.5	4.4	6.0	15.7				
					X-ray	1/2 RUL	Spread	Spread	Clearing	Same	Clearing			
					Enzyme units	130,130	130,117	143,156						
1-26	1700	0.45	i.b.	5-20-33	Bl. cult.	616	408,4	65	0	0			R 4	
					WBC 10.8	16 1	12.6	4.9	11.3	15.6				
					X-ray	1/2 RML	Spread	RML	Clearing	Clearing	Clearing			
					Enzyme units		200,100	100	90					
1-29	1950	0.5	i.b.	6-5-33	Bl. cult.	148	1480	4,174	0				R 4	
					WBC 10.6	21.0	18.5	3.8	26.6					
					X-ray	2/3 RUL	Spread	RUL	Clearing		Cleared			
					Enzyme units	55	55	100						
1-31	1650	0.5	i.b.	6-5-33	Bl. cult.	744	904,190	336,226	20		0		R 6	Sacrificed 8th day Lower 2/3 RLL pneumonia RLL resolving
					WBC 15.4	8.2	4.8	11.6	24.0		34.0			
					X-ray	RLL	RLL	Clearing			Clearing			
					Enzyme units	55	55,150	150,120	120					

No.	Wt.	Dose	Side	Date	Determination	Values	Autopsy / Notes	Code
4-3	1875	0.4	l.t.	5-10-32	Bl. cult.	1010, 160	Animal died suddenly as if choking. Autopsy: Pericarditis; pneumonia: 1/2 RLL, 1/3 RUL	D 2
					WBC 10 0	2 6, 1 1		
					X-ray	1/2 RLL, 1/3 RUL		
					Enzyme units	120		

Pneumonia with septicemia greater than 2000 colonies per cc. In first 3 days after infection

No.	Wt.	Dose	Side	Date	Determination	Values	Autopsy / Notes	Code
2-1	1275	1.0	l.t.	2-8-32	Bl. cult.	4800; 121,000	Autopsy: Fibrinopurulent pleurisy; pericarditis; pneumonia LLL, LML, 2/3 LUL. *B. lactis aerogenes* also present in cultures at autopsy	D 2
					WBC 17 5	3 8; 7,800		
					X-ray	LLL; 2 7, LLL, LML, 2/3 LUL, 130		
					Enzyme units	156		
2-4	1200	0.5	l.t.	2-11-32	Bl. cult.	21,000; 18,000, 0	Autopsy: Empyema, left; pneumonia LLL, 1/2 LML, 1/3 RLL, Culture: IIB-Pn. III, 1520 colonies per cc.	D 1
					WBC 12 2	7.2; 7 0, 1.5		
					X-ray	2/3 LLL, 1/3 RLL; 1/2 LLL, LUL		
					Enzyme units	156; 25		
3-1	1725	0.3	l.t.	3-21-32	Bl. cult.	+; 3200; 4800; 2000	Autopsy: Pneumonia LLL, LML, LUL, RML	D 5
					WBC 19 9	3.3; 6.5; 20.1		
					X-ray	LLL, LML, LUL; Same; Spread RML		
					Enzyme units	25, 25; 25, 12.5		
3-9	1025	0.4	l.t.	4-18-32	Bl. cult.	2800; 25,000; 700; 3750	Autopsy: Fibrinopurulent pleurisy; pericarditis; pneumonia LML, RLL, LLL; congestion LML, RLL	D 4
					WBC 23.9	13.2; 3,730, 7.0, 1.3; 2.2; 6.2		
					X-ray	1/2 LLL; 1/1 LLL; Same; Same		
					Enzyme units	90, 60, 60; 40, 30; 35		
9-1	1900	0.3	l.h.	1-21-33	Bl. cult.	6500; 8	Autopsy: Empyema, right; pneumonia 1/3 RLL; congestion RML, RUL	F. D. 3
					WBC 23.9	20.0; 9.5		
					X-ray	1/2 RLL; Same		
					Enzyme units	400		

TABLE II—*Concluded*

Pneumonia with septicemia greater than 2000 colonies per cc. in first 3 days after infection—*Concluded*

No.	Weight	Dose	Route*	Date		Days after infection 1	2	3	4	5	6	7	Result†	Remarks
9.8	2050	0.33	i.b.	2-27-33	Bl. cult.	3520	2680						F. D. 4	Autopsy: Pericarditis; pneumonia RUL, RML, 1/2 RLL, LML
					WBC 18.3	4.8	2.0							
					X-ray	RUL 1/2 RML LML	RUL RML LML							
					Enzyme units	130, 130	130 143							
1-17	1600	1	i.b.	5-15-33	Bl. cult.	137	2292	3520					D 3	Autopsy: Pneumonia RUL, RML, 1/3 RLL; purulent pleurisy
					WBC 13.2	18.1	20 5	13.3						
					X-ray	1/2 RUL	RUL RML	RUL RML						
					Enzyme units		50	1/3 RLL 50, 50						
1-30	1725	0.5	i.b.	6-5-33	Bl. cult.	33	3800	52, 1000	1056, 188	0			R 5	
					WBC 10 1	18 0	7.9	5.0	5.6	10.3		23.6		
					X-ray	1/3 RUL	1/2 RUL	Clearing	Clearing	Clearing				
					Enzyme units	33	55	110, 150	150, 150					

ment of the same amount was given. On the 4th day resolution was evident in the X-ray. Recovery progressed uneventfully.

Monkey 8-1 (Chart 3). Treatment was begun on the 1st day of the disease, but extension of the pneumonia was noted in the X-rays on the 2nd and 3rd days. On the 4th day resolution was evident. Roentgenograms taken during the course of the disease and recovery are reproduced in Figs. 1 to 6.

M. cynomolgos No. 1-11 ♀ Weight 1950 gm. Route i.b.

	1933 May 15			16			17			18			19			20	
Day of disease				1			2			3			4			5	
Hour	9	1	5	9	1	5	9	1	5	9	1	5	9	1	5	9	11
Blood cult. plate / broth				0 / 0			0			0 / 0			0 / 0				
W.B.C. (thousands)	18.1			28.3			18.2			12.8			12.6				
X-ray	Normal			Lower ⅔ R.U.L.			Entire R.U.L.			Early in R.M.L.?			Clearing				
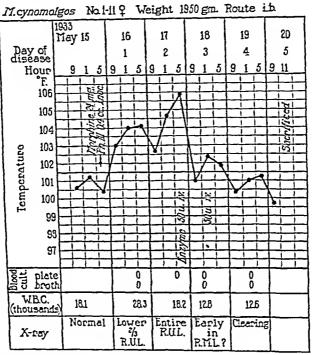
CHART 2. Experimental pneumonia without septicemia, treated with enzyme. *u.* represents units of enzyme.

Group B. Lobar Pneumonia with Septicemia
(1–250 Colonies per Cc.)

In this group of 15 animals the average weight was 1880 gm., the average amount of culture used was 0.37 cc. In 5 cases, two pulmonary lobes were involved, in the remainder only one lobe. Septicemia was present in all instances, the number of organisms present in the blood at the time of treatment ranging up to 166 colonies per 1 cc. of blood in the first 3 days after infection. 3 animals were

treated on the 1st day, 10 on the 2nd, and 2 on the 3rd day after infection. The amount and duration of treatment varied from a single dose of 50 units on the 2nd day, to 5 treatments over the 2nd, 3rd, and 4th days, with a total of 692 units. In one case, No. 1-14, the blood culture contained 14 colonies per cc. on the 1st day, but was sterile on the 2nd day, when treatment was begun. The only other example

CHART 3. Experimental pneumonia treated on 1st day of disease.

of this course of events occurred in Monkey 1-1 of the untreated group, in which septicemia recurred and a fatal termination resulted. In all but 2 cases the pneumonic process was clearly extending at the time of treatment.

Treatment of this group of animals resulted in 100 per cent recovery, in contrast to 55 per cent recovery in the untreated group with comparable septicemia. The average time of recovery was 3.6 days after infection. In 13 of the 15 cases, sterilization of the blood was effected

by the first treatment, in some instances within 4 hours. In one exception, the enzyme was found later to be contaminated, and Type III Pneumococcus was recovered in culture from the blood together with a Gram-negative bacillus. In spite of this, resolution began promptly and continued. In the other case, the blood culture at the time of the first treatment was negative, but a second culture taken $2\frac{1}{2}$ hours later was positive. A third culture on the following day was negative. In another animal, following therapy and while recovery was under way, an intercurrent superficial infection of the extremities was noted, and a Gram-negative bacillus was isolated from the blood.

In 10, or possibly 11 cases, no spread of the pneumonic lesion was demonstrable by X-ray the day following the first treatment; in the majority, resolution was clearly seen. In the 4 cases in which extension of the lesion was noted the 1st day after treatment, there was no further progression as treatment was continued. Concomitantly with the improvement as shown by sterilization of the blood and decrease in the size of the pneumonic process, there was usually a fall in temperature, except in cases in which febrile reactions followed later injections of enzyme. The animals also became stronger and more alert.

In comparison with similar untreated cases (1), the duration of disease in the treated animals was, on the average, almost 1 day shorter than in the untreated animals which recovered, and 2 days shorter than in the fatal cases. Of the untreated cases which recovered, only 2 of 11 presented septicemia on the 1st day of disease, whereas of the 9 fatal cases, 7 had positive blood cultures on the 1st day. The treated cases appear to be more comparable to the latter animals, since of the 15 treated animals 13 had positive blood cultures the 1st day after infection, and, in general, higher septicemias than the fatal cases of the parallel untreated group. The pulmonary involvement was comparable in that progressively spreading pneumonia of lobar distribution was present at the time of treatment. The prompt subsidence of the septicemia and limitation of spread after treatment, together with general improvement, contrasts sharply with the course of the disease in the untreated series.

Monkey 1-18 (Chart 4) represents the type of case in which two pulmonary lobes were involved early in the disease and a relatively high septicemia (60

colonies per cc.) was present. Treatment begun on the 2nd. day resulted in prompt control of the pneumonia, and recovery of the animal.

Monkey 9-2 (Chart 5) illustrates the type of case in which treatment was begun on the 2nd day. The blood, which contained 16 colonies per cc. at the time of treatment, was promptly sterilized, but the X-ray the following day showed some extension of the pneumonia. There is the possibility that the spread

M.cynomolgos No.1-18 ♂ Weight 1900 gm. Route i.b.

	1933 May 15	16	17	18	19	
Day of disease		1	2	3	4	
Hour	9 1 5	9 1 5	9 1 5	9 1 5	9 1 5	
Blood cult. plate / broth		166 +	60	0 0 / 0 0	0	
W.B.C. (thousands)	26	42.8	18	9.2 6	12.8	
X-ray	Normal	Entire R.M.L.	Spread to R.U.L.	Clearing Heart on right	Rapidly clearing	

Temperature graph notes: "Morphine 24 mg. / Ph. III 0.4 cc. i.b.o.s.", "Enzyme 40u. i.b. i.v.", "40u. i.v.", "50u. i.b.", "Remained well"

CHART 4. Effect of enzyme on experimental pneumonia with mild septicemia.

of the lesion seen in the X-ray on the 3rd day occurred in the interval of 6 hours between the time of the X-ray and the time treatment was begun. Nevertheless, recovery rapidly followed.

Group C. Lobar Pneumonia with Septicemia
(250–2000 Colonies per Cc.)

This group comprises 9 animals in which the experimentally induced pneumonia was accompanied by a high septicemia, ranging from the mildest case with 344 colonies per cc. of blood in the first 3 days after infection, to 2 cases with 1700 and 1728 colonies, respectively, during

that period.   Needless to say, the degree of illness in this group was severe.   However, 8 of the treated animals recovered, giving a recovery rate of 88.9 per cent.   This contrasts with the results in untreated animals with similar severity of disease of which only 25 per cent recovered.

The average weight of the animals was 1880 gm.; the average amount of culture injected was 0.39 cc.   In the 1 animal which died, there was involvement of two
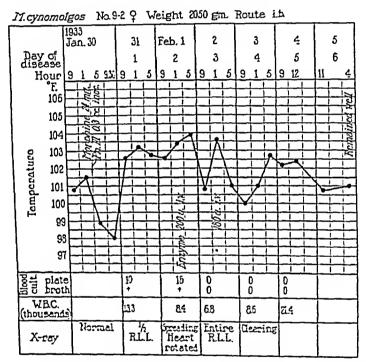
CHART 5. Experimental pneumonia with mild septicemia treated with enzyme.

lobes the morning after infection, and a blood culture yielded 1040 colonies per 1 cc. of blood.   A treatment of 120 units of enzyme was given 4½ hours later. 6 hours after the treatment, the septicemia was reduced to 160 colonies per cc. At this time, the monkey had an attack of choking and died.   A search at autopsy revealed no tracheal obstruction, but definite pneumonic consolidation was present in the right upper and lower lobes, and Type III Pneumococcus was recovered from the pericardial fluid.   The spleen was small and bound by dense adhesions.

general depression and rapidly fatal outcome, clearly unsatisfactory for therapeutic experiments.

Monkey 3-9 (Chart 8) illustrates the effect of enzyme therapy in a case with marked septicemia which terminated fatally. The spread of the pneumonia was checked, a substantial reduction in septicemia was effected, and life prolonged.

M. cynomolgos No. 1-17 ♀ Weight 1600 gm. Route i.b.

CHART 9. Experimental pneumonia with marked septicemia, treated with enzyme but terminating fatally.

At autopsy, the left lower lobe was completely consolidated, while the left middle and right lower lobes appeared congested. Fibrinopurulent pleurisy and pericarditis were present.

Monkey 1-17 (Chart 9) is an instance in which the pneumonia continued to spread, the septicemia increased, and death occurred on the 3rd day after infection. Autopsy revealed lobar pneumonia of the right upper and middle lobes, and partial consolidation of the right lower lobe. A fibrinopurulent pleurisy was present over the involved lobes.

In Monkey 1-30 (Chart 10), which had well marked consolidation at the time of treatment and 3800 colonies of Type III Pneumococcus in culture per 1 cc. of blood, treatment caused prompt limitation of the pneumonia, and two days later sterilization of the blood. Recovery followed. Roentgenograms are shown in Figs. 13 to 18.
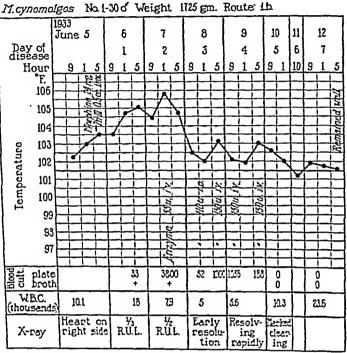
CHART 10. Experimental pneumonia with marked septicemia which recovered after enzyme therapy.

DISCUSSION

The results of specific enzyme therapy in the experimental Type III pneumococcus pneumonia of monkeys have been presented. To evaluate the effects of treatment in the present series of animals, the results, as measured by recovery or death, may be compared with those in an untreated series of animals (1) included in the preceding paper (Table III). It must be borne in mind, however, that in a given experiment the animals which appeared sickest were selected for treatment. Of those animals, treated or not, in which no bac-

terial invasion of the blood was demonstrable, all recovered. In the group with pneumonia accompanied by septicemia of 1 to 250 colonies per cc. of blood, 45 per cent of the 20 untreated animals died, while all of the 15 treated monkeys recovered. Of the 12 untreated animals in the group with septicemia ranging from 250 to 2000 colonies per cc., 75 per cent died, whereas of 9 animals which received enzyme therapy, only 1 died, a mortality rate of 11.1 per cent. The final group includes the animals which suffered from an extremely pronounced form of infection, frequently with diffuse pneumonia, rapid exhaustion of

TABLE III

*The Influence of Enzyme Therapy upon Mortality Rate in Experimental Type III Pneumococcus Pneumonia in Monkeys**

	Untreated				Treated			
Class of infection	No. of animals	No. recovered	No. died	Mortality	No. of animals	No. recovered	No. died	Mortality
				per cent				*per cent*
Pneumonia without septicemia.....	20	20	0	0	8	8	0	0
Pneumonia with septicemia (1–250)..	20	11	9	45.0 ⎫	15	15	0	0 ⎫
Pneumonia with septicemia (250–2000).........................	12	3	9	75.0 ⎬56.2	9	8	1	11.1 ⎬4
Pneumonia with septicemia(2000+).	16	0	16	100.0	8	1	7	87.5
Total........................	68	34	34	50.0	40	32	8	20.0
Total for groups with septicemia...	48	14	34	70.8	32	24	8	25.0

* Classified on the basis of height of septicemia in first 3 days.

circulating leukocytes, septicemia of very high degree, and with a tendency to early death. In the 16 untreated animals of this group, the mortality rate was 100 per cent. Of 8 comparable monkeys to which enzyme was administered, 7 died, and 1, in which the septicemia reached 3800 colonies per cc. before treatment, recovered, a mortality rate of 87.5 per cent.

It can readily be seen that in the groups in which no invasion of the blood occurs, spontaneous recovery is to be uniformly expected, whereas in the extremely severe forms of the disease the great majority of animals are too completely prostrated to respond to any therapeutic

aids. Consequently, the two intermediate groups appear to offer the best opportunity for studying the effects of enzyme therapy. Included in these groups in which septicemia ranged from 1 to 2000 colonies per cc. are 32 untreated monkeys of which 18 died (56.2 per cent), and 24 treated animals with only one death (4 per cent). Or, if one compares the results in all animals in which septicemia was present, 70.8 per cent of 48 untreated animals died, but only 25 per cent of the 32 treated animals.

In addition to the apparently beneficial effects of specific enzyme therapy as measured by survival or death of the animals, certain other favorable influences were observed. In a high percentage of cases in which extension of the pneumonic process was occurring at the time of treatment, the spreading promptly ceased following the initial injection of enzyme. Although the density of the area of consolidation might at first appear greater than before treatment, extension did not occur and resolution of the lesion soon began. This limitation of spread of the pneumonia was not infrequently noted in the severe cases before the bacteria were completely eliminated from the blood stream (Figs. 7 to 12). A comparison of the ultimate degree of pulmonary involvement in the treated and untreated cases (1) (Chart 1) reveals the fact that it was less, in general, in the former series. While in the treated cases the extension was apparently limited early, in the untreated animals extension of the pneumonia progressed, frequently with fatal results.

That the administration of enzyme promoted sterilization of the blood stream seems certain. In the milder cases this occurred quite rapidly. In animals in which the higher degrees of septicemia were present, there was rarely an increase, more regularly a prompt decrease in the number of pneumococci in the blood following the administration of enzyme. Even in cases which eventually terminated fatally, or in which extreme septicemia occurred early in the disease, cultures of the blood showed a marked reduction in the number of bacteria within 4 to 5 hours after the first treatment.

Simultaneously with limitation of the pneumonia, beginning resolution, and elimination of septicemia, a fall in temperature usually occurred. In fact, there was a tendency for the fever to subside concurrently with the cessation of pneumonic spread, even though

septicemia still persisted. Although a marked leukopenia was comparatively frequent at the time treatment was begun, the number of leukocytes rose with the beginning of recovery.

In fatal untreated cases with septicemia, a high incidence of positive cultures was obtained from pleural or pericardial fluids at autopsy. In many instances frank empyema or pericarditis was present. In the treated cases with severe infections which resulted fatally, the incidence of these complications was also high. In recovered animals of the treated series, therefore, a frequency of suppurative complications equal to that of the untreated animals might be expected. The fact that the treated animals which survived recovered without suppurative sequelae suggests that enzyme therapy either prevented the development of empyema and pericarditis or was therapeutically effective even in the presence of these complications.

As previously stated, many technical difficulties have been encountered in attempting to produce enzyme preparations of uniformly high therapeutic activity and purity. The different lots of enzyme have, as a result, been inconstant in both these respects. In some instances toxic effects, attributable to impurities in the material, have been noted in animals after the administration of enzyme. These impurities may induce a febrile reaction and a decrease in the white blood count of the animal. At other times, when the animal is extremely ill with subnormal temperature and a marked leukopenia, the administration of impure preparations may produce a further depression of temperature and of the leukocytes.

The results of the present study indicate that the specific enzyme, even in its present state of purity, exerts a favorable therapeutic effect upon the course and outcome of experimental Type III pneumococcus pneumonia in monkeys. Nevertheless, the present study again emphasizes the therapeutic limitations of the enzyme (4). The action of the enzyme is known to be exerted upon the capsular polysaccharide of Type III Pneumococcus (6). By being deprived of its capsule, the bacterium is made susceptible to phagocytosis by the cells of the animal body. However, when the disease process is of extreme severity and the entire cellular mechanism of the body is markedly depressed, the animal may no longer possess the capacity to dispose of the organisms rendered vulnerable by the specific action of the enzyme.

SUMMARY

The effects of specific enzyme therapy upon experimental Type III pneumococcus pneumonia in monkeys were studied by comparing the course and outcome of the disease in treated animals with that in animals which received no therapeutic aid. Enzyme treatment was found to exert a distinctly favorable influence upon the experimental pneumonia. Treatment was followed by cessation of spread of the pneumonic lesion, sterilization of the blood, and early recovery, except in animals in which the severity of the disease was extreme. While in the untreated animals a high incidence of empyema and pericarditis was observed, suppurative sequelae were apparently prevented by adequate enzyme therapy. The limitations of the therapeutic action of the specific enzyme in the presence of marked depression of the cellular reaction in infected animals are again emphasized.

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EXPLANATION OF PLATES

PLATE 48

Roentgenograms of Monkey 8-1 during the course of experimental pneumonia, treated the 1st day after infection (Chart 3).

FIG. 1. Control. Before inoculation (Dec. 19).

FIG. 2. (Dec. 20.) 19 hours after infection and 7 hours before the first treatment, showing well localized consolidation in lower half of the right upper lobe.

FIG. 3. 2nd day (Dec. 21), showing extension of pneumonia throughout right upper lobe.

FIG. 4. 3rd day (Dec. 22), showing increased density of the shadow over the right upper lobe, but no evidence of further spread.

FIG. 5. 5th day (Dec. 24). Resolution of the pneumonia has begun, as shown by the decrease in density and beginning aeration of the area.

FIG. 6. 10th day (Dec. 29), showing complete resolution of the pneumonic shadow

PLATE 49
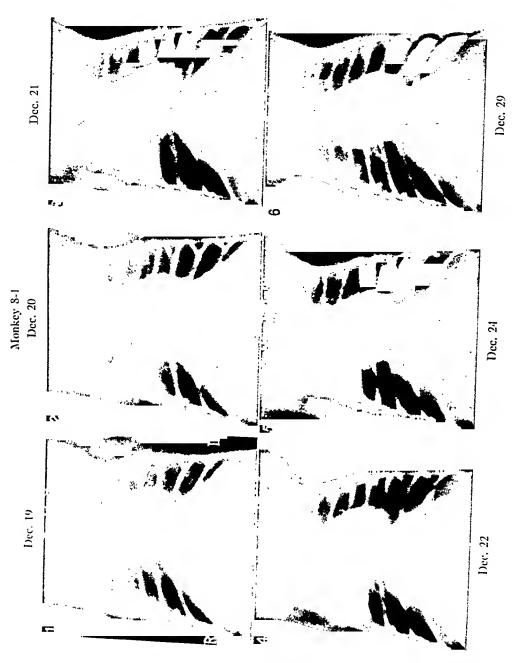
Roentgenograms of Monkey 9-4 during the course of the disease and recovery (Table II).

FIG. 7. 1st day after infection (Feb. 7), showing a well marked early pneumonia of the lower part of the right upper lobe.

FIG. 8. 2nd day (Feb. 8), showing extension of the lesion through the entire right upper lobe, and a small early shadow in the right cardiohepatic angle. Treatment begun.

FIG. 9. 3rd day (Feb. 9). Shows no extension, perhaps some clearing of shadow in right cardiohepatic angle.

FIG. 10. 4th day (Feb. 10). Aeration beginning, as evidenced by the clearly outlined base of heart.

FIG. 11. 5th day (Feb. 11). Base of heart drawn well to right toward resolving lobe, a feature not infrequently noted.

FIG. 12. 1 week later (Feb. 18). Almost complete resolution.

PLATE 50
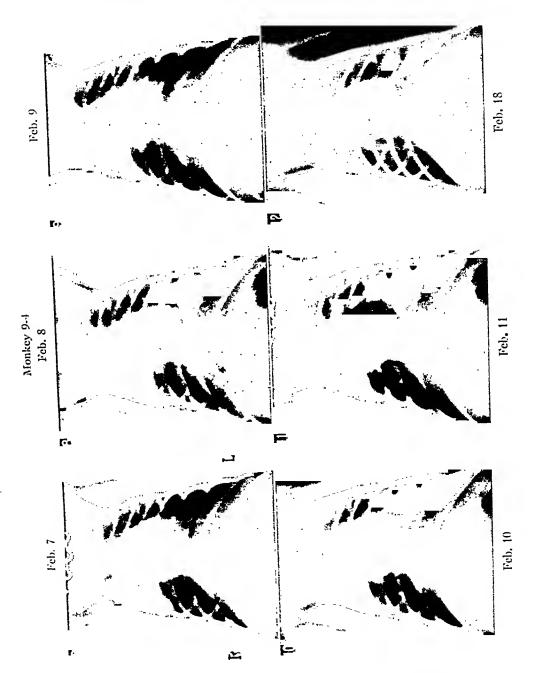
Roentgenograms of Monkey 1-30, first treated on the 2nd day after infection when septicemia had reached 3800 colonies per cc. (Chart 10).

FIG. 13. Control plate (June 5). Shows heart rotated to the right side. Evidence of the extreme mobility of the mediastinum often noted.

FIG. 14. 1st day of disease (June 6), showing early lesion in the lower part of the right upper lobe.

FIG. 15. 2nd day (June 7), showing extension of lesion.

FIG. 16. 3rd day (June 8, following treatment). There is a decrease in density of the shadow, suggesting early resolution.

FIGS. 17, 18. 4th and 5th days (June 9 and 10), showing regression of the lesion.

PLATE 48

Dec. 21

Dec. 29

Monkey 8-1
Dec. 20

Dec. 24

Dec. 19

Dec. 22
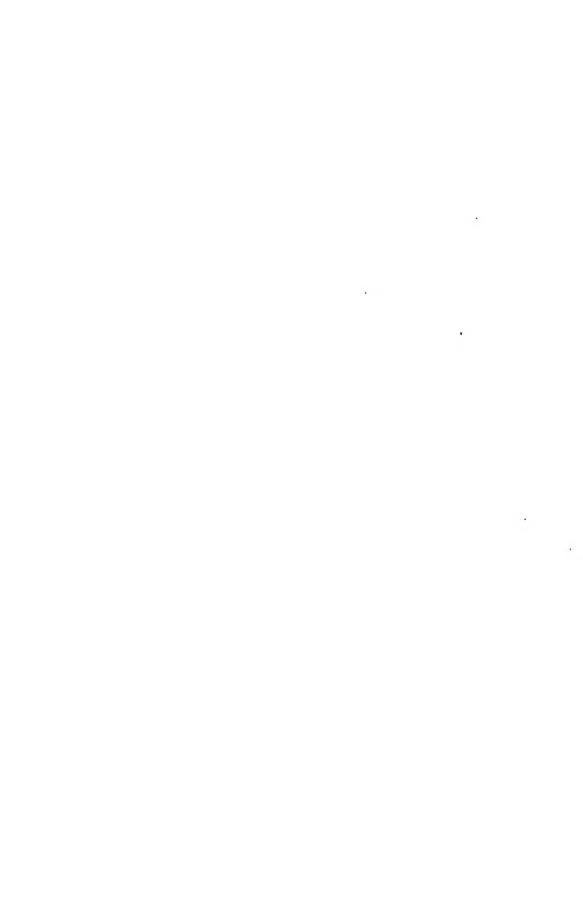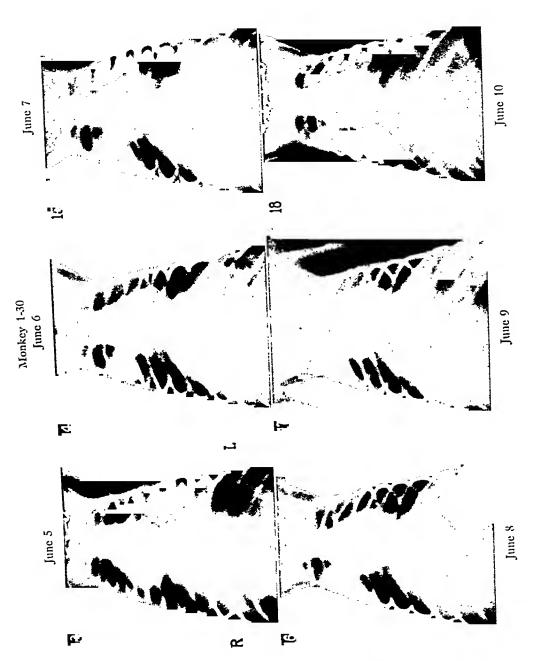
(Francis et al.: Type III pneumococcus pneumonia. II)

(Francis et al.: Type III pneumococcus pneumonia. II)

PLATE 50

LOUPING ILL IN MAN

By THOMAS M. RIVERS, M.D., AND FRANCIS F. SCHWENTKER, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

The fact that the etiological agent of louping ill (1, 2), a natural disease of sheep in Scotland and the northern part of England, is a filterable virus (3) capable of producing in monkeys (4) and mice (5) a disease somewhat similar to poliomyelitis induced us to procure some of the virus for investigation (6). It was supplied to another laboratory of the Institute in which three of the workers, one after another, became sick. Inquiry disclosed the fact that an English investigator had also become ill after having worked with the virus. A preliminary note (7) has already been made regarding these cases. In the present paper a detailed description of the cases and a report of the results of the investigations undertaken to ascertain whether the virus of louping ill was the etiological agent involved are presented.

Report of Cases

Case 1.—Dr. F., male, 28, became sick on Dec. 2, 1932, twelve weeks after the initiation of his work with louping ill virus. For 5 days he experienced general malaise, headache, and a temperature ranging between 101° and 101.5°F. Following this bout of fever the patient was afebrile for 8 days during which time he worked in spite of the fact that he did not feel as well as usual. On Dec. 15, he returned to bed because of a transient diplopia and a temperature of 102°F. From Dec. 15 until Dec. 18, the date of admission to the Hospital, the patient had a headache, fever, and recurring attacks of diplopia, and, in spite of drowsiness, was unable to sleep. On Dec. 17 he experienced several attacks of projectile vomiting which were unaccompanied by nausea.

Upon admission to the Hospital of The Rockefeller Institute the patient had a temperature of 102.1°F. and a pulse rate of 84. He was drowsy and had a headache. The general physical examination was negative with the exception of a marked diminution in the intensity of the deep reflexes and a diplopia caused by a weakness of the internal rectus muscle of the left eye. During the examination it was noticed that the patient's respirations were irregular and accompanied by sighs. There were 10,500 white blood cells per c.mm. of which 83 per cent were granulocytes. On Dec. 19, the day after admission to the Hospital, the patient

continued to have attacks of vomiting but at this time they were accompanied by nausea. No other significant changes were noticed in the patient's general condition. Spinal fluid was obtained for study. It was clear, but contained an increased amount of globulin and 61 white cells per c.mm. of which 88 per cent were mononuclear elements. Cultures of the blood and spinal fluid remained sterile. Mice injected intracerebrally with blood and with spinal fluid remained well. On Dec. 20, the patient received intravenously 50 cc. of a 50 per cent solution of glucose. Two similar intravenous injections were made the next day. Following the administration of the hypertonic solutions the patient's condition
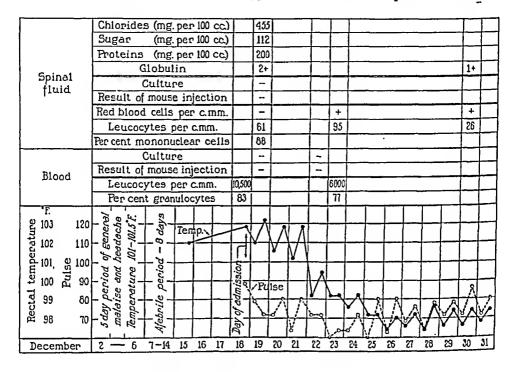
CHART 1. Graphic portrayal of Case 1.

improved; his headache was less severe, the nausea and vomiting ceased, he became jovial and regained the ability to sleep normally. A second specimen of blood was taken on Dec. 22; cultures remained sterile and mice inoculated intracerebrally with small amounts of it remained well. Spinal fluid obtained on the 23rd of December contained blood. That obtained on the 30th had a few blood cells, 26 leucocytes per c.mm., and an increased amount of globulin. The diplopia disappeared on the 22nd. The temperature returned to a normal level on the 23rd. Following this the patient made a rapid recovery since which time he has remained well. See Chart 1 for a summary of the case. At the time the patient was in the Hospital the physicians who saw him believed that he had epidemic encephalitis.

When Dr. F. became sick Mrs. C. carried on the work with louping ill virus until she (Case 2) became sick, Jan. 31, 1933, approximately 6 weeks after she began handling the infectious agent.

Case 2.—Mrs. C., 27, on Jan. 31 went to bed at home because of fever, headache, backache, and prostration. Nothing of significance except a reddened throat was observed by her husband who is a physician. The patient's condition remained unchanged until Feb. 5 when her temperature reached normal. During this period of her illness a marked leucopenia, 2000–4000 cells per c.mm., existed (Chart 2). She gradually improved and returned to work on Feb. 14 at which time the white blood cell count was 6400. On Feb. 18 the headache returned and increased in intensity until the 21st when it became very severe. In spite of the discomfort Mrs. C. continued to work until the afternoon of the 21st when she was carried home prostrated with fever, slight photophobia, and intense headache. On Feb. 22 the white blood cells numbered 12,000 per c.mm. of which 82 per cent were granulocytes. A blood culture taken at this time remained sterile. The fever continued and was accompanied by a slightly confused mental state. On Feb. 23 the patient was admitted to the White Plains Hospital,[1] White Plains, N. Y.

On admission to the Hospital the patient's temperature was 104°F. and the pulse rate was 90. She complained of headache, stiffness of the neck, sore throat, and nausea. Examination revealed that the patient was drowsy and had a stiff neck and an inflamed throat. Feb. 24, she was still nauseated and complained of transient blurring of vision. The white blood cell count was 17,350. Feb. 25, headache, nausea, and vomiting persisted. A consultant was called who, in view of the symptoms, the stiffness of the neck, and the blurring of the optic discs, made a diagnosis of tuberculous meningitis. A lumbar puncture was made. The spinal fluid was clear, reduced Fehling's solution, contained, in addition to an increased amount of globulin, 45 white cells per c.mm. No tubercle bacilli were found in the spinal fluid; cultures remained sterile; mice injected intracerebrally with small amounts of the fluid remained well. The patient's general condition improved rapidly, and, on Feb. 28, her temperature reached a normal level. Although her general condition improved, a definite retrobulbar neuritis developed. Mar. 3, spinal fluid was again obtained for examination. It was slightly blood-tinged; sugar was present; globulin was increased; leucocytes were 50 per c.mm. of which 60 per cent were mononuclear elements; cultures remained sterile. The patient continued to improve and the optic neuritis gradually subsided. Mar. 23, the patient was discharged from the Hospital. After a period of convalescence at home she returned to work and since that time has remained well. Chart 2 summarizes the essential features of the case. In view of the

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[1] Mrs. C. was on Dr. W. W. Mott's service and it was through his courtesy and cooperation that we were able to obtain data regarding her illness.

CHART 2. Graphic portrayal of Case 2.

fact that the patient made a speedy recovery the diagnosis of tuberculous meningitis was changed to acute encephalitis.

When Mrs. C. became sick on Jan. 31, Dr. W. took charge of the work on louping ill. He (Case 3) became ill on Feb. 8.

Case 3.—Dr. W., male, 39, on Feb. 8, after strenuous exercise followed by exposure to inclement weather experienced chilly sensations, dizziness, and elevation of temperature. Feb. 9, he worked in spite of dizziness, anorexia, and a temperature of 101°F. Feb. 10 to 17, he remained in bed because of headache, photophobia, and fever—temperature ranged between 101° and 103°F. Feb. 17 to 20, convalesced at home; headache and weakness persisted. Feb. 20 to Mar. 1, the patient was able to be at the laboratory but was weak and unable to use his eyes for close work. Mar. 1, the patient felt fully recovered and has remained well since that time. The physician in charge of the case made a diagnosis of influenza.

Upon inquiry it was found that an English investigator (Case 4) at the Lister Institute had an attack of encephalitis in 1932 after having worked with louping ill virus. Through the courtesy of Dr. Critchley, 137 Harley Street, London, we have been able to secure the data regarding this patient. The patient himself was in the United States during 1933, a year after recovery from his illness, and we were able to obtain a specimen of serum from him at that time for neutralization tests which will be described later in the paper.

Case 4.—Dr. H., male, 31, from May 10 to 14, experienced headache, soreness in throat, malaise, and muscular stiffness. He worked on May 14 in spite of a headache and a slight elevation of temperature, 100°F. From the 14th until the 19th the patient remained at home because of intense headache, drowsiness, nausea, vomiting, and fever—temperature ranged between 100° and 104.5°F. The pulse was slow. On the 17th the white blood cells numbered 16,000 per c.mm. of which 80 per cent were granulocytes.

In view of the patient's condition he was admitted to King's College Hospital, May 19. Upon admission his temperature was 103.4°F., pulse 64, respirations 20. He was drowsy and had a severe headache; speech was slow; neck was stiff; deep reflexes were diminished in intensity; abdominal reflexes were not obtained. The spinal fluid was under increased pressure and contained sugar, 667 mg. of chlorides per 100 cc., 110 mg. of protein per 100 cc., an increased amount of globulin, 32 red blood cells per c.mm., and 468 leucocytes per c.mm. of which 77 per cent were mononuclear elements. Cultures and smears of the fluid revealed no microorganisms. On May 20, 21, and 22, lumbar punctures were made (Chart 3), and the spinal fluid was found to be of the same general nature as that already de-

Spinal fluid	Chlorides (mg. per 100 cc.)	667	660	640	660					718	
	Sugar (mg. per 100 cc.)	—	Present	—	—					Pres.	
	Proteins (mg. per 100 cc.)	110	200	330	440					320	
	Globulin	4+	Trace	—	—					1+	
	Culture and smear									—	
	Red blood cells per c.mm.	32	1440	368	118					0	
	Leucocytes per c.mm.	458	740	304	218					66	
	Percent mononuclear cells	77	88	96	99					97	
Blood	Culture		—								
	Leucocytes per c.mm.	16,000	13,200								
	Per cent granulocytes	80	82								

Day of admission.

4 day period of headache, sore throat, malaise, and muscular stiffness.

Temp.— Pulse —○

CHART 3. Graphic portrayal of Case 4.

scribed. The presence of blood cells should be noted, inasmuch as it was believed not to be due to the trauma of the puncture. In fact, several specimens of the fluid were brownish in color. May 20, the patient's general condition was improved; his fever was not as high as it had been; the tendon and abdominal reflexes were absent; the optic discs showed decided pallor. May 21, the patient's temperature reached the normal level and remained there. He felt better, was rational, and took a small amount of solid food. A marked tremor of the face, eyes, tongue, and hands was noticed. This persisted for several days and gradually disappeared. Tendon and abdominal reflexes were still not obtained. May 25, patient was entirely free from headache, was very alert, and wanted to get up. May 26, deep reflexes were still absent, but the abdominal reflexes had returned. The patient continued to improve, but on June 3 he developed a definite weakness of the muscles of the upper and lower parts of the left side of the face and some difficulty was experienced in blinking the left eyelid. Nothing else abnormal was noted. In fact, all the reflexes had returned to a normal state of activity. A lumbar puncture made at this time revealed a clear colorless fluid which contained sugar, 718 mg. of chlorides per 100 cc., 320 mg. of protein per 100 cc., an increased amount of globulin, no red blood cells, and 66 leucocytes per c.mm. of which 97 per cent were mononuclear cells. Cultures and smears of the fluid showed no bacteria. From June 3 until the time of discharge from the Hospital, June 18, the patient improved rapidly; the facial weakness gradually decreased and finally disappeared during convalescence at home. Since this illness the patient has been well. See Chart 3 for a summary of the significant features of the case. At first it was thought that the patient had tuberculous meningitis, but, in view of his rapid recovery, a final diagnosis of acute encephalitis was made.

EXPERIMENTAL

There is no record in the literature of the occurrence of louping ill in man, yet the disease was considered in connection with the cases we have reported, and attempts to demonstrate the virus in the blood and spinal fluid of two of them were made without success. Failure to demonstrate the virus under such conditions, however, was not considered proof that the patients had not had louping ill, because it is not always possible to demonstrate the virus in the blood and spinal fluid of monkeys known to have the disease. Consequently, inasmuch as we had developed a test (6) for the presence of neutralizing or protective antibodies in the sera of monkeys immune to louping ill, we decided to apply the test to the sera of the four individuals who were suspected of having had the disease. In addition to these sera, we also tested the sera of other people working with the virus, as well as the sera of a number of individuals who had had no known contact

with the active agent. The manner in which the tests were conducted will be described. Then, several of them will be presented in the form of tables. Finally, a summary of the results will be presented.

Method of Conducting the Neutralization Tests

The virus emulsions used in the neutralization experiments were prepared from pooled brains of mice killed at the height of a louping ill infection. After removal from the animals, the brains were stored for 24 hours in separate containers in an ice box while bits of each were tested by means of cultures for the presence of ordinary bacteria. The brains free from bacteria were pooled and ground in a mortar. Sufficient Locke's solution was then added to make a 20 per cent emulsion which was centrifuged at 2000–3000 R.P.M. for 10 minutes. Then decimal dilutions of the supernatant fluid were made with Locke's solution. Portions of each dilution were mixed with an equal amount of the serum the neutralizing properties of which were being investigated. The mixtures were allowed to stand for 2 hours at room temperature and then for 1 hour in a refrigerator at 0°C. 0.03 cc. of each of the mixtures were then injected intracerebrally, respectively, into each of six mice. The animals were observed for 18 days, and the number of deaths and the day of death of each animal were recorded. No mouse that succumbed sooner than the 4th day after injection was considered to have died of louping ill.

In each test at least two control sera were used. These consisted of a serum (negative control) that did not neutralize the virus and of a neutralizing serum (positive control) either from an immune monkey or from an immune person. It must be remembered that one cannot use virus emulsions diluted with Locke's solution alone as negative controls, because it has been shown (6) that in emulsions diluted in such a manner the virus of louping ill deteriorates rapidly. The Rockefeller Institute strain of albino mice was employed in approximately one-third of the experiments, while the Swiss strain of albino mice was used in the others. The latter mice are very susceptible to the virus and are highly suitable for this type of work. The former are not 100 per cent susceptible to the active agent, yet they proved satisfactory for the tests even though the results obtained with them were not so striking as were those when Swiss mice were used.

Results of Neutralization Tests

In the manner described above 17 tests were conducted in which sera from 63 individuals were examined for the presence of neutralizing antibodies for louping ill virus. In Table I, Test 2 is summarized, and from the results presented it is obvious that the sera from Individuals 1 and 8 are comparable to that of the negative control, Individual 19, while the sera from Individuals 5 and 6 are comparable

TABLE I
Summary of Neutralization Test 2

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Immune monkey serum plus virus dilutions (positive control)					
10^{-3}	6	0			
10^{-4}	6	0			
10^{-5}	6	0			
	18	0	0		
Serum of Individual 1 plus virus dilutions					
10^{-3}	6	5		7, 7, 7, 8, 9	
10^{-4}	6	4		6, 6, 7, 10	
10^{-5}	5	5		7, 8, 9, 10, 10	
	17	14	82		8.0
Serum of Individual 8 plus virus dilutions					
10^{-3}	6	6		6, 6, 8, 8, 8, 12	
10^{-4}	4	2		7, 8	
10^{-5}	5	3		7, 8, 10	
	15	11	73		8.0
Serum of Individual 5 plus virus dilutions					
10^{-3}	5	1			
10^{-4}	6	0			
10^{-5}	6	0	12		
	17	1	6		12.0
Serum of Individual 4 plus virus dilutions					
10^{-3}	6	0			
10^{-4}	5	0			
10^{-5}	6	0			
	17	0	0		
Serum of Individual 19 plus virus dilutions (negative control)					
10^{-3}	6	4		6, 8, 10, 10	
10^{-4}	5	3		7, 7, 10	
10^{-5}	5	3		7, 10, 12	
	16	10	63		8.8

Rockefeller Institute mice used in the test.

TABLE II

Summary of Neutralization Test 17

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Immune monkey serum plus virus dilutions (positive control)					
10^{-4}	6	2		9, 11	
10^{-5}	4	0			
10^{-6}	6	0			
	16	2	13		10.0
Serum of Individual 6 plus virus dilutions					
10^{-4}	6	0			
10^{-5}	6	0			
10^{-6}	6	0			
	18	0	0		
Scrum of Individual 2 plus virus dilutions					
10^{-4}	6	0			
10^{-5}	6	0			
10^{-6}	6	0			
	18	0	0		
Scrum of Individual 38 plus virus dilutions					
10^{-4}	6	4		8, 9, 9, 9	
10^{-5}	6	2		10, 11	
10^{-6}	6	1		13	
	18	7	39		9.4
Scrum of Individual 17 plus virus dilutions					
10^{-4}	6	6		7, 7, 8, 8, 8, 9	
10^{-5}	6	6		7, 7, 8, 9, 9, 11	
10^{-6}	6	6		8, 8, 9, 9, 9, 10	
	18	18	100		8 4
Scrum of Individual 7 plus virus dilutions					
10^{-4}	6	6		7, 7, 7, 8, 8, 9	
10^{-5}	6	6		7, 8, 8, 8, 9, 9	
10^{-6}	5	5		6, 8, 9, 9, 9	
	17	17	100		8.0

TABLE II—*Concluded*

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Serum of Individual 45 plus virus dilutions					
10^{-4}	6	6		7, 8, 8, 8, 9, 9	
10^{-5}	6	6		7, 8, 9, 9, 9, 9	
10^{-6}	6	6		7, 8, 9, 9, 9, 9	
	18	18	100		8.4
Serum of Individual 43 plus virus dilutions (negative control)					
10^{-4}	5	5		7, 7, 8, 8, 8	
10^{-5}	6	6		6, 7, 8, 8, 9, 9	
10^{-6}	6	6		7, 9, 9, 9, 9, 10	
	17	17	100		8.0

Swiss mice used in the test.

to that of the monkey immune to louping ill. Table II summarizes Test 17 and reveals (1) that the sera of Individuals 7, 17, and 45 are comparable to that of the negative control, Individual 43; (2) that the sera of Individuals 2 and 6 are comparable to that of the monkey immune to louping ill; (3) that the serum of Individual 38 possesses less neutralizing antibodies than do the other positive sera.

In Table III a summary of all the neutralization tests is presented. The sera for this work were collected from both sexes and from individuals 1 year to 68 years old. The majority of the people, however, were in the neighborhood of 35 years of age. A number of the sera were tested more than once and more than one specimen of serum was collected from six of the individuals. The sera from the different individuals were given numbers which have been arranged in the table according to whether the persons from whom the sera were collected had had close contact (Nos. 1–7), possible contact (Nos. 8–17), or no history of contact (Nos. 18–63) with the virus of louping ill. In addition to this arrangement, the nationality of the individuals, the diseases from which they were suffering or from which they had recently recovered are indicated. Finally the results—percentage of deaths of mice used—of the neutralization tests and our interpretation of them are given.

TABLE III
Summary of Neutralization Tests

Person's No.	Nationality	History of contact with virus	Notes on person	Percentage of mice that died			Interpretation of results of neutralization tests
				Negative control	Positive control	Test serum	
1	American	Close contact	Normal	63	0	82	−
				100	6	100	
				100	0	100	
				78	0	72	
2	"	"	"	72	0	29	+
				100	0	38	
				100	0	12	
				88	0	0	
3	"	"	Recovered from acute encephalitis	100	19	37	+
				100	13	0	
				67	12	6	
				88	0	0	
4	English	"	"	63	0	0	+
				88	0	12	
				86	0	14	
				88	0	12	
5	American	"	"	63	0	6	+
				88	0	0	
				88	0	0	
6	"	"	Recovered from headache and fever	100	0	25	+
				100	0	0	
				88	0	0	
				100	13	0	

No.	Nationality	Contact	Diagnosis			
7	American	Close contact	Normal	100	13	100
8	"	Possible contact	"	83	0	63
9	"	"	"	72	0	72
10	"	"	"	78	0	72
11	English	"	"	67	11	72
12	"	"	"	94	28	100
13	American	"	"	74	6	78
14	"	"	"	100	0	100
15	"	"	"	100	0	100
16	"	"	"	100	0	100
17	"	"	"	75	6	100
18	"	"	"	100	13	100
19	"	No history of contact	"	100	0	100
20	Australian	"	"	100	0	82
21	Chinese	"	"	63	28	83
22	"	"	"	78	0	100
23	"	"	"	100	0	88
24	"	"	"	86	0	100
25	Egyptian	"	"	86	0	100
26	"	"	"	88	0	100
27	"	"	"	100	0	100
28	South American	"	"	100	0	100
29	"	"	"	100	0	100
30	"	"	"	100	0	100
31	American Negro	"	"	100	0	100
32	"	"	"	100	0	86
33	"	"	"	100	0	86
34	"	"	Syphilis of aorta	88	0	100
35	"	"	Arteriosclerosis	88	0	100
36	"	"	Pseudobulbar palsy	100	0	
37	"	"		100	0	

TABLE III—*Concluded*

Person's No.	Nationality	History of contact with virus	Notes on person	Percentage of mice that died			Interpretation of results of neutralization tests
				Negative control	Positive control	Test serum	
38	American Negro	No history of contact	Obesity, pulmonary edema	88	0	12	+
39	American	"	Epidemic encephalitis	100	19	35	−
40	"	"	"	100	13	39	−
41	"	"	"	100	11	100	−
42	"	"	"	100	11	100	−
43	"	"	Postinfection encephalitis	100	17	84	−
44	"	"	Encephalitis?	100	17	67	−
45	"	"	Benign lymphocytic meningitis	100	0	100	−
46	"	"	Pertussis, vaccinia	100	11	88	−
47	"	"	Varicella	78	0	100	−
48	"	"	Measles, vaccinia	100	13	100	−
49	"	"	Measles	78	17	100	−
50	"	"	Measles, pneumonia, vaccinia	78	28	71	−
51	"	"	Pneumonia	88	17	100	−
52	"	"	"	86	28	83	−
53	"	"	Aplastic anemia	88	28	71	−
54	"	"	"	88	0	88	−
55	"	"	Rheumatic fever	86	0	88	−
56	"	"	"	86	0	100	−
57	"	"	"	100	0	86	−
58	"	"	"	88	0	75	−
59	"	"	Rheumatic heart disease	88	0	75	−
60	"	"	Carcinoma of stomach	100	0	100	−
61	"	"	Hypertension	86	0	88	−
62	"	"	Nephritis	86	0	83	−
63	"	"	"	88	0	86	−

+ indicates that the serum of the individual neutralized the virus of louping ill.

− indicates that neutralization did not occur when the individual's serum and the virus were mixed.

An examination of Table III reveals the following facts: No evidence was obtained to indicate that nationality and race determine the presence or absence of neutralizing properties of serum for louping ill virus. The sera of individuals sick of or recovered from nephritis, hypertension, arteriosclerosis, syphilis, carcinoma of the stomach, rheumatic heart disease, rheumatic fever, aplastic anemia, pneumonia, measles, pertussis, varicella, vaccinia, pseudobulbar palsy, epidemic encephalitis, postinfection encephalitis, benign lymphocytic meningitis, do not neutralize the virus of louping ill. Although the age and sex of the persons from whom the sera were collected are not shown in the table, it can be stated that these factors have no apparent effect upon the presence or absence of significant neutralizing antibodies against the active agent. The striking fact obtained from a study of the results in the table is that close contact of individuals with the virus is in some manner associated with the appearance in their sera of neutralizing properties. For instance, of seven people who had been in close contact with the active agent, five possess neutralizing sera. Of these five, three (Cases 1, 2, 4) had had encephalitis, one (Case 3) had been sick with an influenza-like disease, and one had not been consciously ill while working with the virus. Of ten individuals who might have had contact with the active agent but who had not worked with it, none possesses a neutralizing serum. Of 46 people who gave no history of contact with the virus, only one, No. 38, possesses a serum with neutralizing antibodies and these upon repeated tests were found to be less active or less abundant than were those of the other positive sera. There is no obvious explanation of why this serum should have been positive.

<div style="text-align:center">DISCUSSION</div>

Several interesting features of the cases presented in the first part of the paper deserve comment. It should be noted (Charts 1, 2, 3) that all of the patients had a pulse-temperature disproportion and that a small amount of blood was present in a number of the specimens of spinal fluid which the operators believed was not due to the trauma of the needle. One patient was thought to have had epidemic encephalitis, two were considered to have had tuberculous meningitis. Since all of them recovered promptly and completely it is unlikely that these

diagnoses were correct. All of the physicians who examined the patients, however, agree that the individuals had an encephalitis. Two (Cases 1, 2) of the four patients had an influenza-like disease followed by a short period of fair health before the onset of symptoms and signs of encephalitis. A third patient (Case 4) was sick for 4 days, although he continued to work, before the onset of the severe symptoms of encephalitis. The fourth patient (Case 3) had what seemed to be nothing more than a severe attack of influenza. It has been stated (8) that louping ill virus may at times produce in sheep an infection without much apparent involvement of the central nervous system. Consequently, one wonders whether the primary illness in three (Cases 1, 2, 4) of the patients and the illness of the fourth (Case 3) represent systemic infections which in three instances were followed by involvement of the central nervous system. In sheep and monkeys, ataxia due to involvement of the cerebellum is a prominent feature of louping ill. The cases presented in this paper evidenced no signs of ataxia. This fact, however, does not preclude the possibility that they represent instances of the disease in man.

The question of whether the attacks of disease in man described by us represent instances of infection with louping ill virus now arises. In fact, one might suggest that they represent cases of encephalitis similar to those that occurred in St. Louis during the summer of 1933. This is not true, however, because sera from our cases, which neutralize the virus of louping ill, do not neutralize the St. Louis virus (9). Unfortunately, a definite answer to the question raised cannot be given, because louping ill virus was not recovered from any of our cases. Nevertheless, the circumstances under which they occurred and the results of the neutralization tests make it likely that they represent such an infection. If the cases represent louping ill infections in man, the frequency with which they occurred in one laboratory was probably due to the fact that the intranasal instillations of the virus in large numbers of mice, practiced in that laboratory, led to the exposure of the workers to large doses of the virus suspended in the air in droplets of moisture.

The situation described by us is somewhat unique, because louping ill virus has been in America for only a short period of time, and, so far as is known, is to be found in only four laboratories. Therefore, the

facts obtained by means of our neutralization tests may be of significance in relation to certain general phenomena of immunity and for this reason may be interpreted in a broader way than merely as an attempt to diagnose the cases presented. For example, recently it has been suggested (10) that the neutralizing antibodies against poliomyelitis virus in the sera of adults who have not had obvious attacks of poliomyelitis are due to serological maturation instead of contact with or of subclinical infections with the virus. From the results of our experience with neutralization tests in connection with louping ill virus it appears that the antibodies which we demonstrated are not likely to arise in the absence of the active agent.

SUMMARY

Four instances of infection in man which are believed, because of the circumstances under which they occurred and in view of the results of neutralization tests, to represent cases of louping ill have been described. Evidence obtained by the neutralization tests is in favor of the idea that the antibodies against louping ill virus demonstrated in certain sera were most likely the result either of contact with or of infection with the active agent.

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THE EFFECT OF CARROT FEEDING ON THE SERUM PROTEIN CONCENTRATION OF THE RAT*

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In a previous paper (1) it was suggested that the eating of large amounts of carrots might be followed by a lowering of the plasma proteins and the indirect evidence favoring this hypothesis was analyzed. The present experiments were designed to test the question more directly.

Material and Methods

The methods used in the previous experiments were followed in the present work. In brief, young but mature white rats (150–220 gm.) previously on an adequate stock ration were placed on the experimental diet. After various intervals groups of four to six animals were exsanguinated under ether anesthesia, aliquot parts of serum from each were pooled and the total serum proteins were measured by the gravimetric method of Barnett, Jones and Cohn (2).

The Experimental Diet.—In order to reduce the problem to its simplest terms the diet consisted exclusively of carrots. The oxheart variety which is said to have a high content of carotene (3) was used although there was no special reason for this selection other than convenience. The carrots always came in fresh and had not been in storage. A liberal ration was placed in the cages every 2nd day and it was estimated that each rat ate 60–100 gm. or more daily. Denton and Kohman (4) studied the nutritive properties of carrots and pointed out that they contain approximately 1 per cent of protein. Each rat, therefore, ingested about a gram (or a little less) of protein daily; how much of this was digested and absorbed, and to what extent carrot protein is adequate for rats we cannot state. Carrots are said to contain considerable quantities of water-soluble and fat-soluble vitamins as well as salts (Na, Ca, Cl, P, Fe). Theoretically, therefore, they afford a ration not obviously inadequate from a nutritional standpoint, and in fact Denton and Kohman reported that rats on an exclusive carrot diet (except for the addition of salts) remained in apparently good health for as long as 16 weeks.

RESULTS

The animals seemed to thrive on the carrot diet and they remained lively and ate well throughout the experiment even when ascites developed. Not a single animal died during the course of the observations which extended over 21 weeks. There were none of the usual evidences of vitamin deficiency; the hair, skin and nails and eyes remained normal, and there were no signs of gastrointestinal or respiratory tract disorder. The dystrophy of the hair which was previously noted in rats on a diet low in protein but otherwise adequate was not encountered in the present experiments.

There was a prompt and progressive loss of weight (Figs. 1 and 2) for the first 7 weeks, after which there was only a gradual decline. The significance of the figures is somewhat obscured first by the huge amount of gastrointestinal content of the carrot-fed rats together with an actual hypertrophy of the tract and later (see below) by the development, in some animals, of collections of fluid. The whole gastrointestinal tract (including contents) of the carrot rats weighed from 25–40 gm. as against 10–25 gm. in the controls. The contents weighed 10–15 gm. as against 3–6 gm. in the controls and the tract itself weighed 20–30 gm., an increase over normal of about 10 gm., probably due to work hypertrophy.

No gross pathological changes were evident in the viscera except that the livers, in a few of the rats with ascites, appeared to be fatty— they were pale and yellowish and the normal markings were indistinct. The mesenteric fat was well preserved in all but one animal.

No signs of subcutaneous edema were noted. After the first few weeks, however, the tissues (peritoneum, fascia, cut muscle) seemed moist in contrast to the controls. Five rats developed frank ascites and hydrothorax (see Table I).

Changes in the Serum Proteins

The total blood volume as measured by exsanguination decreased as the animals lost weight. Because of the complicating factors of fluid accumulation and variable gastrointestinal residue no accurate correlation with weight could be made. The changes in concentration of serum proteins are shown in Table II and Fig. 3. It is seen that there was a progressive lowering as time went by. The lowest figure
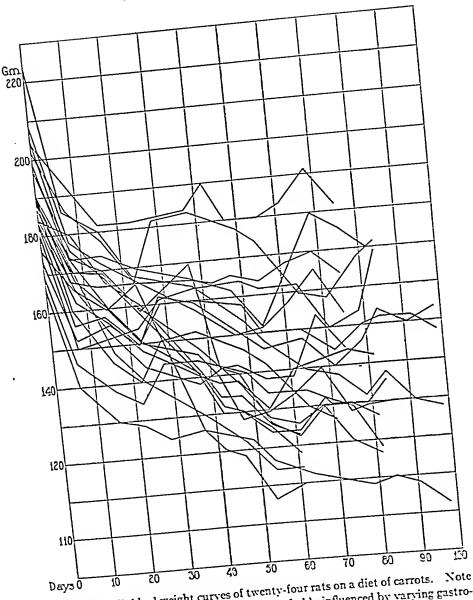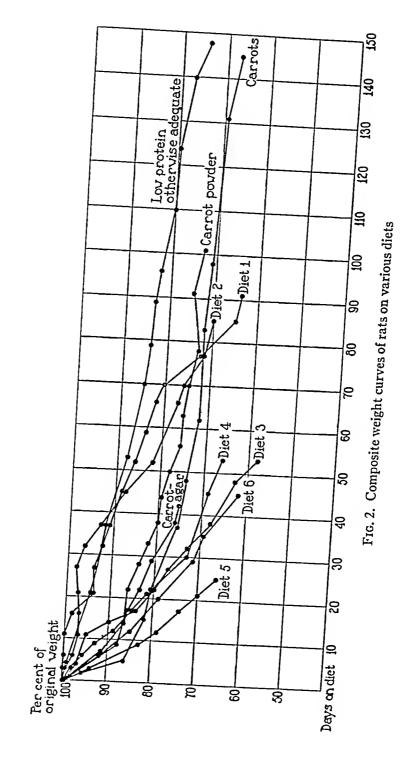
FIG. 1. Individual weight curves of twenty-four rats on a diet of carrots. Note the irregular shape of the curves which are probably influenced by varying gastro-intestinal content and accumulation of fluid.

FIG. 2. Composite weight curves of rats on various diets

for pooled serum from a group of rats was 4.28 gm. per cent on the 145th day, but in individual animals (see Table I) values as low as 2.99 were obtained.

Explanation of the Changes

It may be accepted as a fact, then, that the concentration of serum protein falls in rats on an exclusive carrot diet and our next task is to attempt to explain this phenomenon. The point at issue, of course, is whether carrots exercise some positive serum protein-lowering in-

TABLE I

The Development of Ascites in Rats on an Exclusive Carrot Diet

Rat No.	Duration of carrot feeding	Initial weight	Final weight	Corrected weight	Weight loss	Serum proteins	Remarks
	days	*gm.*	*gm.*	*gm.*	*per cent*	*gm. per cent*	
19163	82	186	170	130	30	—	Ascites = 20 cc. Hydrothorax = 5 cc.
19161	82	204	126	110	46	—	Ascites = 3 cc. Hydrothorax = 1 cc.
19160	61	180	120	105	42	4.00	Ascites = 3 cc.
19131	63	172	132	100	40	2.99	Ascites = 15 cc. Hydrothorax = 7 cc.
19190	145	190	134	109	43	—	Ascites = 17 cc. Hydrothorax = 8 cc.

fluence, and clearly no such influence can be assumed until other and simpler explanations have been excluded.

Is the Hypoproteinemia Due to an Inadequate Protein Ration?— In a previous paper (1) we showed that rats kept on a diet which afforded not more than 0.2–0.3 gm. of protein daily showed no significant drop in serum protein concentration over a period of 5 months. With the carrot diet the daily protein ration per rat was about 1.0 gm. It seems unlikely, therefore, that lack of protein alone explains the drop of serum protein in the carrot rats although it is by no means
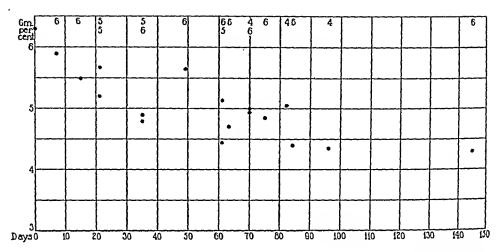
FIG. 3. Relation of serum protein concentration of rats to the duration of carrot feeding. The number of rats in each group is indicated at the top of the chart.

TABLE II

Serum Proteins of Groups of Rats on Exclusive Carrot Diet

Length of time on diet	No. of rats in group	Per cent of original weight	Per cent of original weight (corrected)	Total serum protein of pooled serum of group
days				*gm. per cent*
Untreated controls				6.30–6.50
7	6	93	87.2	5.90
14	6	93	87.1	5.49
21	5	90.2	84.5	5.67
21	5	85.6	80.6	5.21
35	6	84.0	79.0	4.80
35	5	83.8	77.9	4.88
49	6	88	82.9	5.66
61	5	62.9	56.0	4.44
61	6	82.6	77.4	5.13
63	6	68.0	60.6	4.72
70	6	75.5	70.4	4.95
70	4	74.5	69.4	5.00
75	6	78.2	73.2	4.86
82	4	83.8	78.2	5.06
98	4	71.2	67.0	4.34
145	6	67 0	50.5	4.28

certain that the animals actually absorbed an adequate amount of protein complete for their needs.

Is the Hypoproteinemia Due to Loss of Protein in the Urine?—12 hour total urines were collected from two lots of six rats each which had been on the carrot diet for 6 weeks. The average output of protein per rat for the two groups was 0.39 mg. and 0.67 mg. per 100 sq. cm. of body surface—values two to three times normal (5) but evidently inadequate to account for a significant lowering of serum protein.

Is the Hypoproteinemia Due to Vitamin Deficiency or to Some Other Factor Which Renders the Carrot Diet Inadequate?—Regardless of theoretical considerations as to its nutritional value, the exclusive diet of carrots was, in fact, inadequate in the present experiments as evidenced by the rapid loss of weight which the rats underwent. A study of Fig. 3, furthermore, shows that while the serum protein level bore a general relationship to the duration of carrot feeding there are rather gross discrepancies. Two lots of rats both killed on the 61st day, for example, showed serum protein values of 4.45 and 5.13 gm. per cent respectively. These discrepancies are even greater if individual rats are considered. One wonders, therefore, whether the fall of serum protein after carrot feeding is a specific phenomenon or whether the eating of any inadequate diet which leads to marked loss of weight will not result in a similar hypoproteinemia especially if the diet is low in protein.

To control this possibility rats were placed on various obviously inadequate diets (see below), the weight curves were studied and the serum proteins were measured. Weight loss was rapid in every case (Fig. 2) and since the experience of this laboratory has been that death occurs when the fat stores are depleted the animals were sacrificed when their weight fell to the vicinity of 100 gm., which was considered to be the danger zone. Aside from weight loss the animals appeared lively and normal and no lesions were found at autopsy. No visible edema or serous sac effusion developed in any of these rats in distinction to those fed on carrots.

Diet 1.—Starch-lard mixture (starch 75 per cent, lard 25 per cent) 90 per cent, casein 10 per cent. This diet is defective in protein and in vitamins. There was rapid loss of weight (Fig. 2). Four rats were killed on the 85th day and four on

the 92nd day. The serum proteins of the two groups were 4.98 and 5.46 gm. per cent—an average of 5.22.

Diet 2.—Starch-lard mixture 90 per cent, yeast 5 per cent, alfalfa 2 per cent, casein 3 per cent. This diet is defective in protein, but better supplied with vitamins than Diet 1. However, as with Diet 1 weight loss was rapid (Fig. 2). Five rats were killed on the 71st day and five on the 85th day. The serum proteins of the two groups were 5.26 and 5.20 gm. per cent respectively—an average of 5.23.

Diet 3.—Starch 70 per cent, lard 11 per cent, casein 5 per cent, salt mixture 4 per cent, cod liver oil 10 per cent. Weight loss was very rapid (Fig. 2) on this diet. The five animals were sacrificed on the 53rd day. The serum proteins were 5.57 gm. per cent.

TABLE III

Weight Loss and Serum Protein Level of Rats on a Diet of Starch and Lard

Length of time on diet	No. of rats	Weight (per cent of original)	Serum protein
days			*gm. per cent*
16	6	84.9	5.62
19	6	74.4	5.13
28	5	67.8	5.14
35	7	69.5	5.00
35	6	67.4	4.86
42	6	67.6	5.22
45	6	64.2	4.55

Diet 4.—Starch-lard mixture 95 per cent, casein 5 per cent. The weight curve is shown in Fig. 2. Ten rats were killed on the 47th day, five on the 53rd day. The serum proteins of the two groups were 5.44 and 5.55 gm. per cent.

Diet 5.—Starch-lard mixture 99 per cent, casein 1 per cent. The weight curve is shown in Fig. 2. Ten rats were killed on the 25th day. The serum proteins were 5.59 gm. per cent.

Diet 6.—Starch 75 per cent, lard 25 per cent. This diet was not only devoid of protein but grossly ill balanced and defective in regard to vitamins and salts. On it rats lost weight rapidly (Fig. 2) but there was no other evident departure from health. No edema developed. A good many animals were placed on this diet and the results are summarized in Table III.

In summary, then, rats on defective Diets 1 to 6 lost weight quite rapidly. In spite of the fact that they were not killed until death was imminent (as judged by weight loss) no animal in the whole series became edematous. Furthermore with the exception of some of the

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rats on Diet 6 the serum proteins dropped little if at all below the level of about 5.25–5.50 gm. per cent which we regard as physiological for rats on any sort of low protein diet (1). In no case, with the exception of some of the rats on Diet 6, did the proteins fall to the levels commonly encountered in rats on the carrot diet.

It must be emphasized that Diets 1 to 6 do not serve in the true sense as controls for the carrot diet, since a proper control diet would be one on which the animals live as long and in as good condition as those on the test diet. From this standpoint the diet low in protein, but otherwise adequate which we previously studied (1), is our best control. On it there was no lowering of serum proteins comparable to that which occurred with the carrot regimen. With Diet 6 there was, to be sure, a considerable fall of the serum protein level but this diet

TABLE IV

Total Serum Proteins of Rats Fed Exclusively on Dry Carrot Powder

No. of rats	Length of time on diet days	Total proteins of pooled serum gm. per cent
		6.30–6.50
	0	5.12
Control	57	5.53
9	84	5.76
6	101	
4		

is so inadequate from every standpoint that rats cannot be maintained on it for more than a few weeks, and hence no definite conclusion can be drawn as to whether the lowering of serum proteins was due to lack of protein or to general malnutrition.

Is the Hypoproteinemia Related to the Large Bulk of Water in the Carrot Diet?—Since the previous observations gave no definite answer to our question, further experiments were carried out in which rats were fed not fresh carrots, but dry carrot powder.

A commercial preparation was used which contained (by our own analysis) 391 mg. per cent of nitrogen which could account for a maximum protein content of 2.5 gm. per cent. Nineteen rats were used in this experiment. The carrot powder, and no other food of any sort, was offered, and water was allowed ad lib. Each rat ate on the average about 10 gm. per day containing not over 0.25 gm. of protein. The animals throve and when the last batch was sacrificed

on the 101st day they appeared entirely normal except for loss of weight. There was no suggestion of edema or ascites and the organs were small but they appeared normal. There was considerable loss of fat. The composite weight curve is shown in Fig. 2 and the serum protein estimations are given in Table IV.

It is seen that there was practically no lowering of serum protein on the carrot powder diet in contrast to that obtained with fresh carrots. Since the obvious difference between the two was water content it was decided to do further experiments with carrot powder,

TABLE V

Results of Feeding 10 Per Cent Carrot Powder Suspended in 3 Per Cent Agar-Agar

Rat No.	Duration of artificial carrot feeding	Ascites	Hydrothorax	Serum protein
	days			*gm. per cent*
24162	23	2.0 cc.	Few drops	
24166	23	11.0 cc.	2 cc.	
24175	23	Few drops	—	
24179	23	0.5 cc.	—	4.54
24264	23	2.0 cc.	Few drops	
24279	23	0.5 cc.	—	
24176	42	0.5 cc.	—	
24178	42	2.0 cc.	Few drops	
24154	42	2.0 cc.	—	
24153	42	2.0 cc.	Few drops	4.64
24177	42	2.0 cc.	—	
24186	42	2.0 cc.	—	
24163	42	1.0 cc.	—	
24161	42	0.5 cc.	—	

but to make the rats take as much water as they obtained with the whole carrot diet. To this end an artificial carrot was constructed as follows:

The best grade of agar-agar was made up in 3 per cent solution in distilled water, and after cooling to about 60°C., 10 gm. of carrot powder were added to each 100 cc. The mixture was stirred in a mechanical kitchen mixer so as to get uniform distribution of the powder and it was then allowed to solidify in deep dishes. After hardening it was cut into blocks and placed in the feed boxes. Each animal ate from 50–100 gm. of this carrot-agar ration daily and hence ingested a large quantity of water. The composite weight curve shown in Fig. 2

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is similar to that of the rats fed on fresh carrots. There were no signs of the ordinary nutritional disorders which result from vitamin insufficiency. No recognizable subcutaneous edema was noted, but every rat in the series developed free fluid in the peritoneum and the blood proteins dropped rapidly to definitely subnormal values. The results on the whole paralleled those obtained with the fresh carrot diet but were more extreme and more constant. They are summarized in Table V. At autopsy, aside from decrease in peritoneal fat, the organs looked normal, though small. The gastrointestinal tract was hypertrophied as in the carrot rats and contained a huge agar residue.

These experiments show that addition of water (and agar) to the carrot powder serves to reproduce the effects which follow a diet of fresh carrots. It seems therefore that carrots contain no specific substance effective in lowering blood proteins and producing edema. Why the ingestion of water should produce these effects is not yet clear and further work on this point is in progress.

DISCUSSION

It has been shown that rats subsisting on a diet of carrots remain in good general condition for periods of as long as 21 weeks. There is, however, loss of weight, at first rapid, later more gradual, and a fall in the concentration of serum proteins. When this drop is extreme (4.50 gm. per cent or lower) ascites and hydrothorax are likely to develop. The response of individual rats varies greatly, however, even when they are all under similar conditions, so that loss of weight and drop of serum protein concentration occurs much more rapidly in some animals than in others.

The point at issue in the interpretation of the experiments is whether carrots contain some agent which has a positive disturbing effect on the blood protein-regulating mechanism or whether the drop in serum proteins is a non-specific effect of malnutrition. The fact that controls on a variety of low protein, ill balanced, vitamin-deficient diets failed to develop edema and suffered for the most part only a slight drop of serum proteins, if any, below the physiological level suggests that carrots actually exercise a positive deleterious influence. Further analysis however has shown that a diet of dried carrot powder leads neither to hypoproteinemia nor edema (ascites), whereas the forced addition of water by suspending the powder in agar reproduces all the effects of fresh carrot feeding. Water, therefore, seems to be the cru-

cial factor rather than some specific constituent of carrots. Incidentally it may be noted that the artificial carrot offers a simple and certain method of producing hypoproteinemia in an animal otherwise in good nutritive condition, and we propose to use this technique for the study of further aspects of the subject.

Finally it is of interest to correlate the carrot hypoproteinemia with clinical malnutritional disorders. It appears that a combination of factors is necessary in both cases; namely, an inadequate total caloric intake, an ill balanced diet, a defective protein ration and a large fluid intake. No one of these factors alone seems adequate to produce hypoproteinemia, at least with any constancy.

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THE EFFECT OF A GROWTH-PROMOTING EXTRACT OF THE ANTERIOR PITUITARY ON THE EARLY GROWTH OF THE ALBINO RAT*

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Data are here presented on the weight and water content of the various organs and parts of castrated male albino rats as influenced by the injection of an anterior pituitary growth-promoting extract during the greater part of the first 2 months of life. The data were accumulated in the course of another investigation (1) in which it was shown that a growth-promoting extract of bovine anterior pituitary does not prevent the increase in gonad-stimulating content of the pituitary of the rat which occurs after castration (2, 3). It was felt that the data on the water content would throw additional light on the question of the composition of the tissues of rats and mice injected with anterior pituitary extract as reported recently by several investigators (4–8), since these investigators in their analyses used the animals *in toto* without attempting, as indeed the magnitude of the task would forbid, to follow the changes in the separate parts and organs. In addition, it was thought that the data on the weight of the different organs and parts of the animals might yield some interesting information as to the early effects of an excess of anterior pituitary growth-promoting principle on the development of the young organism that is still rapidly growing.

Inspection of the literature as to the effects of castration on the growth of the albino rat indicates that the castration of the animals of the early age here employed has not materially altered the results from what they would have been had non-castrates been used. Stotsenburg (9), using three different groups of male albino rats castrated

on the 14th or 15th day of life and carried up to the 143rd, 181st, and 185th day of life, respectively, found that the growth curve for the castrates was practically the same as that for the normal animals. Hatai (10) castrated his animals at weaning or shortly after and reported that the castrates did tend to become smaller in weight and length than the non-castrate controls, but this difference was but slight after approximately 5 months. Van Wagenen (11) found like Hatai that male albino rats castrated at weaning did not maintain the weight and body length of their unoperated controls, but this failure was not always evident during the first 100 to 150 days of life; after this time there was an increasing divergence. Van Wagenen's graphs of the average weight curves for castrates and non-castrates indicate that there was no divergence at all in the weight of her animals during approximately the first 50 days of life.

The rats here employed were castrated at weaning and killed when the difference in weight between pituitary-injected and control groups had just become manifest. This was when the animals were approximately 56 days old (youngest litter 53 days, oldest 59). It may be assumed, then, with some degree of confidence that so far as total body weight is concerned there was at this age but little difference, if any, in the growth of the castrates from what it would have been had they been normal animals.

Material and Methods

The growth-promoting extract was prepared in accordance with the method described by Bugbee, Simond, and Grimes (12). This method is essentially as follows: Whole beef pituitaries are used which have been placed on ice at the packing house as soon as possible after the death of the animals. The anterior lobes are dissected free from the surrounding tissues and shelled out. The resulting anterior lobe tissue is ground as fine as possible and to this hash is added for every 100 gm. of tissue 400 cc. of 0.05 N sodium hydroxide containing 2.5 per cent butanol. The mixture is put away to stand in the refrigerator overnight. The pH of the mixture is then brought down to 7.2 to 7.6 by the addition of 0.2 N acetic acid. Centrifugation for ½ hour throws down the insoluble residue. The cloudy supernatant liquid is warmed to 37°C. on the water bath and to every 100 cc. is added 20 gm. of sodium sulfate. Again centrifugation for ½ hour separates precipitate and supernatant liquid. The precipitate is dissolved in an amount of 0.02 N sodium hydroxide containing 2.5 per cent butanol so that 2 cc. of the resulting solution contains the material extracted from 1 gm. anterior lobe tissue

(200 cc. of the sodium hydroxide solution for the precipitate from 100 gm. tissue). The gland residue remaining from the first extraction may be re-extracted with one-half the volume of sodium hydroxide used in the first extraction, and the sodium sulfate precipitation from this second extract added to the solution of the first extract. The resulting fluid is filtered through asbestos three times and finally through sterile porcelain filters into suitable sterile containers, such as small glass ampoules or bottles. The material is then kept in the refrigerator until used.

A total of 120 albino male rats was accumulated consisting of 30 litters, 4 mates to a litter. The animals had at all times access to food and water. Each litter was weaned on the 21st day of life and castrated on the same day. Injections were begun the same day or the day following, and were given subcutaneously, 0.5 cc. twice daily. 1 animal in each group, A, received the growth-promoting extract. A 2nd animal in each group, B, received beef muscle extract prepared in the same way as the preceding. The other 2 animals remained as untreated controls. The injections were continued until the animals were, on the average, 56 days old. At this time 3 animals from each litter were killed. These consisted of the one receiving the growth-promoting extract, the control receiving the beef muscle extract, and that one of the untreated controls which weighed the most. The latter animal thereby served as an untreated age control, AC. The smallest untreated control was allowed to grow until it had reached approximately the same gross weight as had its litter mate receiving the growth-promoting extract, at which time it was killed. This animal thereby served as a weight control, WC.

The animals were disposed of as follows: Each animal was etherized, and through a midline incision the gut was drawn out exposing the inferior vena cava. With a large-bore needle and syringe, blood was withdrawn from the vena cava until the heart had stopped beating. The vena cava was then transected and the remainder of the blood which came out and could be forced out by appropriate pressure was immediately taken up with weighed cotton swabs. The swabs were quickly placed into a weighing bottle kept closed, and when all swabs had been collected in the bottle they were reweighed. Likewise the needle and syringe were weighed before and after collecting the blood. The gut was freed of its mesenteric attachments from esophagus to anus and discarded. The bladder was emptied of any urine present, using a small needle and syringe. The edges of the wound were brought together and the animal was weighed. To this weight was added the weight of the blood previously removed, to give what was called the final weight of the animal. The animal was now stretched on its back on a sheet of white paper, the position of nose and anus marked off, and the distance between the marks measured. The average of several measurements for each animal was taken. The organs and parts were removed in the following order: spleen, adrenals, kidneys, liver, thymus, heart, lungs, thyroid, skin, tail, pituitary. The non-furry portion of the skin was retained on the tail. The small amount of blood which came out following severance of the vessels to the organs was as before taken up with cotton swabs and weighed, and this weight added to that of the blood previously removed. Each organ when removed was placed into a

weighing bottle containing filter paper previously weighed. The paper served to prevent the moisture of the organ from wetting the bottle; also, it served as a vehicle, by grasping one corner with a forceps, for transferring the organ back and forth. The adrenals, thyroid, and pituitary when removed were washed in physiological saline, quickly dried on filter paper, and weighed directly on a torsion microbalance. The posterior lobe of the pituitary was plucked away immediately after the whole gland had been weighed, and the anterior lobe reweighed alone to give the weight of the posterior lobe by difference. The organs, still on the filter paper, were placed in clean beakers to dry in the oven at 100°C., and when dry were reweighed in the weighing bottles. The skin and carcass were placed to dry in the oven in previously weighed beakers. Corresponding portions of litter mates were put in and removed from the oven at the same time. The so called constant weight was taken to be the point where the organs first began losing only a few (1 to 3) mg. in 24 hours, since with organs of a size such as is found in the rat such a loss may keep up for days. The average length of time in the oven was for the smaller organs 5 days, for the larger 7 days.

In the treatment of the data, the method given by Fisher (13) is used because of its theoretical advantage over the usual method as far as the experimental set-up here employed is concerned. The usual method for determining whether or not the difference between two means is significant involves the use of the standard deviations of the means. Fluctuations in the value of the standard deviation of a mean may be readily brought about by the introduction of items which deviate widely from the mean. In biological work, wider variations may be expected to exist between non-litter mates than between litter mates. It is theoretically possible, therefore, in an experiment in which the control group is made up of litter mates of the animals in the experimental group, that small but constant differences existing between each experimental animal and its litter mate control would be obscured by the wider variations between the non-litter mates in each group if the mean of the one group as a whole be compared with the mean of the other group as a whole. Fisher's method obviates this possibility by dealing directly with the differences existing between litter mates. For ease of calculation Fisher's formula is recast as follows:

$$ t_{A,B} = \frac{\bar{\Delta}_{A,B}}{\sqrt{\dfrac{\Sigma(\Delta_{a,b})^2 - n(\bar{\Delta}_{A,B})^2}{n(n-1)}}} \quad * $$

---

* The t of Fisher has the same meaning as the $\dfrac{x}{\sigma}$ of other statisticians but is more inclusive in that in its table for its distribution he includes those cases where n is small (30 or less) whereas the usual tables for the distribution of $\dfrac{x}{\sigma}$ apply only where n is large (30 to ∞).

where $\Delta_{a,b}$ = difference between any two litter mates a and b in Groups A and B,

$$\bar{\Delta}_{A,B} = \frac{\Sigma \Delta_{a,b}}{n} = \text{mean of algebraic sum of all the differences,}$$

and n = number of differences between litter mates.

Since this formula does not involve the use of the standard deviation, this is not given except for the data in Table I. Appended to the original data in each table are the so called P values which indicate whether or not the difference between any two means is to be regarded as significant.[1]

RESULTS

Table I shows that the extract was potent in producing animals heavier and longer than the beef-injected and untreated age controls. (E.g., the P value 0.0001 for the difference between the means A and B indicates that there is but 1 chance in 10,000 of obtaining by random sampling alone a difference as great as the one observed; hence the difference is undoubtedly a significant one.) The weight controls are in turn heavier than the pituitary-injected group, but in nose-anus length these two groups are the same. The P value 0.06 for the difference in nose-anus length between beef-injected and untreated age controls suggests that the former animals are somewhat smaller than the latter.

Table II presents the average group weights of the various parts and organs. The data on the blood are the averages from 22 animals in each group instead of 30, the blood from the first 8 animals in each group having been discarded without weighing. The results are best summarized as follows:

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[1] A P value of 0.01 for the difference between any two means indicates that there is but 1 chance in 100 of obtaining again by random sampling alone a difference as great as the one observed. Customarily, the P value 0.01 is taken as a limit on one side of which (0.01 or less) it can be said that the difference observed is undoubtedly significant. Values between 0.01 and 0.05 indicate lesser degrees of significance which must be interpreted with reference to other factors in the experiment. Values greater than 0.05 are not generally considered as indicating that a significant difference is present. This is a safe and conservative procedure to follow. Here, however, because of the variety and interlocking of the data, values as high as 0.08 have been interpreted as indicating a tendency toward a significant divergence where it was felt that there was a sound physiological basis for such an interpretation. The P values given in the tables here were obtained by calculating from Fisher's table of t a table of P corresponding to values of t ranging by tenths from 0.1 to 6.0, for $n = 1, 2, 3 \ldots 20$ and ∞.

1. Adrenals, thymus, and spleen show no significant differences in weight throughout the four groups.

2. The weight of the heart and skin of the beef-injected animals in Group B is significantly decreased in comparison to that of the untreated age controls in Group AC. This adds considerable strength to the suggestion seen in Table I that the animals in Group B tend to be somewhat smaller in nose-anus length than those in Group AC.

TABLE I

Average Final Weight and Length of Animals

	Body weight	Nose-anus length
	gm.	*mm.*
Group A (pituitary-injected)..................	146.7 ± 23.0	185.8 ± 8.7
Group B (beef-injected)......................	129.9 ± 15.4	178.6 ± 6.2
Group AC (untreated age controls).............	133.9 ± 20.7	180.5 ± 7.6
Group WC (untreated weight controls)..........	151.4 ± 22.9	185.1 ± 9.2
A, B	0.0001*	0.0001
A, AC	0.002	0.0001
A, WC	0.002	0.23
B, AC	0.16	0.06
B, WC	0.0001	0.0001
AC, WC	0.0001	0.003

* This means that there is but 1 chance in 10,000 of obtaining again as a result merely of the sampling process a difference between the means for Group A and Group B as great as the one here observed.

3. The increased weight of the pituitary-injected animals in Group A over that of the untreated age controls in Group AC is reflected in the case of the heart, lungs, liver, kidneys, blood, tail, and probably also the carcass.

4. Heart, lungs, liver, kidneys, and blood of the pituitary-injected animals exceed in weight the corresponding organs of the weight controls.

5. The increased weight of the weight controls in Group WC over the pituitary-injected animals in Group A is manifested chiefly in the carcass and probably also in the skin; too, the thyroid and pituitary of this group are heavier than in the pituitary-injected.

TABLE II

Average Fresh Weights of Parts and Organs

(a) Endocrines

	Adrenals	Thyroid	Whole pituitary	Anterior lobe pituitary	Posterior lobe pituitary
	mg.	mg.	mg.	mg.	mg.
Group A	26.6	20.2	7.0	5.6	1.5
Group B	25.3	19.1	7.9	6.7	1.6
Group AC	27.0	20.3	8.2	6.8	1.7
Group WC	26.3	23.3	9.0	—	—
A, B	0.19	0.16	0.0001	0.002	0.08
A, AC	0.42	0.84	0.0001	0.003	0.04
A, WC	0.76	0.001	0.0001	—	—
B, AC	0.11	0.11	0.16	0.77	0.44
B, WC	0.05	0.0001	0.0001	—	—
AC, WC	0.92	0.0007	0.0003	—	—

(b) Viscera

	Thymus	Spleen	Heart	Lungs	Liver	Kidneys
	gm.	gm.	gm.	gm.	gm.	gm.
Group A	0.574	1.314	0.714	0.977	9.712	1.418
Group B	0.600	1.229	0.611	0.848	7.387	1.206
Group AC	0.560	1.294	0.645	0.912	7.660	1.218
Group WC	0.588	1.298	0.661	0.929	8.088	1.303
A, B	0.84	0.62	0.0001	0.0001	0.001	0.0001
A, AC	0.19	0.84	0.0001	0.02	0.0001	0.0001
A, WC	0.55	0.69	0.0001	0.02	0.001	0.0002
B, AC	0.32	0.42	0.009	0.13	0.19	0.69
B, WC	0.76	0.32	0.004	0.09	0.05	0.04
AC, WC	0.19	0.76	0.48	0.76	0.16	0.01

(c) Parts

	Blood	Tail	Skin	Carcass
	gm.	gm.	gm.	gm.
Group A	10.3	5.699	31.94	84.77
Group B	8.5	4.936	28.47	76.44
Group AC	8.7	5.140	31.18	80.35
Group WC	9.5	5.707	33.67	89.36
A, B	0.0002	0.0001	0.01	0.003
A, AC	0.0002	0.0001	0.29	0.08
A, WC	0.0008	0.62	0.07	0.0004
B, AC	0.49	0.09	0.02	0.11
B, WC	0.008	0.0001	0.002	0.0002
AC, WC	0.01	0.001	0.03	0.0008

6. The pituitary gland in the pituitary-injected animals is smaller in weight than in any of the other groups. Only after this trend of the data from the first half of the animals was noticed were the separate weights of the two lobes determined, so that the averages of the two lobes pertain to 16 animals in each group instead of 30. This number is sufficient to show that the decrease in weight of the anterior lobe is unquestionable. In view of this, it is probable that the posterior lobe is also decreased in weight, but a larger number of animals would be necessary to make this difference in weight as definite as it is for the anterior lobe.

TABLE III

Average Water Content of Parts and Organs (Per Cent of Fresh Weight)

	Thymus	Heart	Lungs	Spleen	Liver	Kidneys	Tail	Skin	Carcass
Group A............	79.9	78.8	80.4	77.1	72.0	74.4	62.8	60.6	71.9
Group B...........	80.1	78.6	80.4	78.4	71.7	73.9	62.3	58.1	72.5
Group AC.........	80.1	78.3	80.1	77.9	72.2	73.9	62.2	55.4	72.2
Group WC........	79.4	78.3	79.6	78.0	73.2	73.8	60.8	55.2	71.7
A, B	0.84	0.76	0.37	0.13	0.76	0.004	0.19	0.008	0.07
A, AC	0.76	0.48	0.62	0.84	0.84	0.002	0.16	0.0001	0.5
A, WC	0.009	0.32	0.0001	0.32	0.19	0.003	0.0001	0.001	0.5
B, AC	0.76	0.72	0.69	0.32	0.48	0.76	0.76	0.01	0.33
B, WC	0.002	0.84	0.0001	0.48	0.48	0.48	0.0002	0.02	0.004
AC, WC	0.0001	0.92	0.06	0.48	0.48	0.62	0.0001	0.92	0.09

Table III gives the average water content of the various parts and organs. The pituitary-injected animals show a significant increase in water content of kidneys and skin in comparison to the other groups. However, the skin of the beef-injected animals shows also an increased hydration in comparison to the skin of the age controls and weight controls. The weight controls show less water than all the other groups in thymus, tail, and lungs.

In the attempt to analyze further the nature of the increased hydration of the skin and kidneys of the pituitary-injected animals the data of Table IV were worked up. This shows that the increased water content of the kidneys of the pituitary-injected group is not solely responsible for the increased weight of these organs since there has

been at the same time a definite increase in dry matter. But this is not so in the case of the skin. Here the dry weight in the pituitary-injected group tends to be less, if anything, than in the untreated age controls; it is about the same in amount as in the beef-injected group, which similarly shows a decreased amount of dry matter in comparison to the age controls. In other words, both pituitary-injected and beef-injected animals show the same type of change in the skin with respect to the untreated age controls: an increase in the water content and a decrease in the amount of dry matter.

TABLE IV

Dry Weight of Kidneys and Skin

	Kidneys	Skin
	gm.	gm.
Group A..................................	0.327	12.67
Group B..................................	0.282	11.94
Group AC.................................	0.286	14.02
Group WC................................	0.308	15.13
A, B	0.0001	0.29
A, AC	0.0001	0.02
A, WC	0.005	0.003
B, AC	0.48	0.01
B, WC	0.005	0.001
AC, WC	0.005	0.12

DISCUSSION

Of the investigators referred to at the beginning of this paper, Downs and Geiling, Bierring and Nielsen, and Lee and Schaffer agree that the tissues of pituitary-injected animals show an increased hydration in comparison to controls. Wadehn alone finds that his injected mice show no increase of water in their tissues. According to Wadehn, this difference is due to the fact that he prepares the growth principle by precipitation from extracts which have been carefully dialyzed free from electrolytes. This method, he feels, yields a preparation purer than that used by previous investigators, and he attributes the hydration effect obtained by Downs and Geiling to the presence in their extracts of an "antidiuretic" principle.

The extract used here is one that is essentially similar to the ones used by investigators other than Wadehn, and their finding that such an extract causes an increased hydration is confirmed. This increased hydration, however, is shown here to be confined to the skin and the kidneys, and is not a generalized affair.

Moreover, it is probable that in the case of the kidneys the increased hydration is only apparent. It does not seem likely that we can be dealing here with a true interstitial or intracellular retention of water. The histological structure and functional nature of the kidney makes it difficult to conceive of such an occurrence. More likely, what was measured here was really an increased amount of urine in the kidney tubules subsequent, no doubt, to an increased food and water intake. That the food intake of the pituitary-injected animals was greater than in the weight controls is suggested by the fact that despite the care taken to kill off the weight controls when they had reached exactly the same gross weight as the pituitary-injected animals, the net weight of the weight controls after the gut had been stripped away was greater than that of the pituitary-injected animals, indicating that the gut contents of the latter must have been greater. On the other hand, part of this difference in weight may have been due to an increase in size and weight of the gut itself.

At any rate, it appears that actually there is but one organ in the pituitary-injected animals that shows an increased amount of water and that organ is the skin. Moreover, this increased hydration has been produced without an increase in total weight of the skin in comparison with the weight of the skin in the untreated age controls. It seems highly improbable that the same principle that would cause this type of change in the skin would effect the increase in weight without an accompanying increase in hydration that is observed, for example, in the case of the heart. Further, there is in the increased hydration of the skin of the beef-injected animals the suggestion that at least part of the skin hydration of the pituitary-injected animals may be non-specific.

All in all, it appears that the data of this experiment might reasonably be interpreted as favoring Wadehn's view that the increased hydration observed in the tissues of animals injected with anterior pituitary extracts is not due to the growth principle. But the question as to

whether or not the hydration is produced by a specific water balance principle remains unsettled, since there is evidence that at least part of the increased hydration of the skin noted here is due to a non-specific factor or factors.

The fact that the beef-injected animals show in comparison to the untreated age controls a tendency toward a smaller nose-anus length and a significantly smaller heart and skin suggests that the beef-muscle extract exerted a mildly deleterious effect on the growth of the animals in this group.

The decreased weight of the pituitary gland in the pituitary-injected animals may be explained as due to the fact that the parenteral injection of the growth principle rendered unnecessary the production by the gland cells of as much of the principle as they would otherwise have had to produce so that we are dealing here at least to some extent with a process akin to the atrophy of disuse.

By the use of weight controls in this experiment it is demonstrated that the increase in weight of the heart, lungs, liver, and kidneys of the pituitary-injected animals constitutes a true splanchnomegaly, since these organs in this group are significantly heavier than the corresponding organs in the weight controls despite the fact that the total body weight of the latter is greater. It has thus been possible to reproduce experimentally during what might be termed the prepuberal or early puberal life of the rat two characteristics of hyperpituitarism, visceral and skeletal overgrowth, which are in man not seen until the onset of adult life. As far as I am aware, the simultaneous production of these two consequences of an excess of anterior pituitary substance at so early an age has not hitherto been reported.

How is the increase in weight of the blood removed from the pituitary-injected animals to be interpreted? It may simply mean, of course, that the vessels in these animals were more dilated than they were in the controls so that more blood could be removed by the method employed. More attractive, however, is the hypothesis that it represents actually an increase in the amount of the vascular bed, since from this hypothesis one may draw the inference that this increase in vasculature may be a primary effect of the growth-promoting principle, and the visceral and skeletal growth secondary to this. The actual meaning of the phenomenon remains, of course, to be

demonstrated by further experimentation, and work is now under way in which is being studied in greater detail the effects of anterior pituitary extracts on the blood and cardiovascular system.

SUMMARY

Castrate male albino rats were injected with a growth-promoting extract of bovine anterior pituitary from the 21st day of life (day of castration) to the 56th day (average for group) at which time the difference in weight between the pituitary-injected animals and controls was first clearly discernible. In comparison to controls the pituitary-injected animals at this stage showed: (1) a decrease in the weight of the pituitary gland; (2) an increase in nose-anus length; (3) an increase in the weight of heart, lungs, liver, and kidneys which is shown to be of the nature of a splanchnomegaly; (4) an increase in the weight of the blood removed; (5) an increase in the water content of the skin and the kidneys; (6) a tendency toward an increase in weight of the carcass. The significance of these findings is discussed.

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OBSERVATIONS ON THE BLOOD CYTOLOGY IN EXPERI-MENTAL SYPHILIS

I. The Period of Disease Activity

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Among the outstanding features of the blood picture in experimental syphilis of the rabbit, Pearce (1) has reported the following: A slight increase in the total white count; unchanged or lowered lymphocyte values during the earlier phase of the disease, with increased values during the period of regression and healing of lesions; and a marked increase in the number of monocytes during the period of disease activity. These findings were presented in terms of percentage deviation from preinoculation mean values, and the results, which were considered from the standpoint of the clinical course of the disease, were charted with particular reference to the time in weeks after inoculation.

With these observations as a background, and in connection with our investigations of various aspects of host reactions to environmental influences, including disease conditions, the study of the blood of syphilitic rabbits was continued. Special attention was again paid to the cytological levels during the active phase of the disease as compared with preinoculation values, while additional comparisons were made with the findings in normal rabbits during the same period. Furthermore, observations were also made in the period of latency when all clinical manifestations of the disease have healed and the animal appears to be "normal." The results of this study are reported in this and the subsequent paper (2). In the latter paper (2), a comparison is made between the cytological findings in experimental and human syphilis (3).

Material and Methods

The rabbits employed were all purchased from dealers and were of the types commonly utilized in laboratories, that is, grays, browns, and Flemish crosses. At the beginning of the experiment, they averaged 4 to 6 months of age. Each animal was housed in an individual cage in a well ventilated room receiving sunlight. The diet consisted of hay, oats, commercial food pellets, and a free supply of water.

Weekly blood examinations of 20 normal rabbits were begun on October 2 and continued to November 13, 1930; on this date the 20 animals were compared as to weight, color, physical condition, and blood formula, and a balanced division was made into an experimental and a control group of 10 animals each. On November 14, 1930, the animals comprising the experimental group were inoculated in the right testicle with 0.3 cc. of a saline emulsion of an actively growing syphilitic lesion from an animal infected with the Nichols strain of *Treponema pallidum*. Weekly blood examinations of the two groups were continued to February 26, 1931, when the main experiment was terminated. Selected animals were, however, retained for other studies. Before the termination of the experiment, three of the inoculated animals were utilized for tissue studies (4) and one of the control group died of an intercurrent infection. These four animals were withdrawn from present consideration and the results, therefore, comprise observations on the blood cytology of an experimental group of seven and a control group of nine animals.

Each blood examination consisted of a total red and white cell count made with standardized pipettes and a differential count of 100 cells by the neutral red supravital technique. The Ringer-heparin method of Casey and Helmer (5) was employed for such platelet counts as were made. At the start of the experiment, hemoglobin estimations were made by the Newcomer method, but these are not reported here because a routine check of our hemoglobinometer revealed slight inaccuracies. All animals of the two groups were examined on the same day and the examinations were conducted on the same day of consecutive weeks.

For the purpose of analysis, two periods were considered: The *preinoculation* period from October 2 to November 13, 1930, during which there were seven counts on each animal, and the *postinoculation* period from November 14, 1930, to February 26, 1931, with 15 counts on each animal (Table I). The results are analyzed on the basis of all counts made on the experimental and control groups during these two periods. The mean values for all the blood elements of each group were determined for each period from all the counts on the particular group during the particular time interval. The following comparisons were made: (1) The mean values for 49 counts on the experimental group during the preinoculation period were compared with the mean values for 63 counts on the control group for the same period. (2) The mean values for the 49 counts on the experimental group during the preinoculation period were compared with the mean values for the 105 counts on the same group during the postinoculation period. (3) The mean

values for the 63 counts on the control group during the preinoculation period were compared with the means for the 135 counts on the same group during the postinoculation period.   (4) A last comparison was made between the mean values in the postinoculation period for the 105 counts on the experimental group and the 135 counts on the control group.   At the time these experiments were begun, platelet counting was not a routine part of our hematological procedure and platelet determinations were not made every week.   We have, however, 19 platelet counts on the experimental group and 27 counts on the normal control group made on the same days of 3 consecutive weeks in the preinoculation period. These are compared in the same manner as the other blood elements with 42 determinations on the experimental group and 54 on the control group made on the same days and during the same 6 weeks of the postinoculation period.

The usual statistical procedures were employed for calculating the standard

TABLE I

Analysis of Blood Examinations

Group	Preinoculation period Oct. 2 to Nov. 13, 1930			Postinoculation period Nov. 14, 1930, to Feb. 26, 1931		
	No. of animals	No. of examinations	No. of counts	No. of animals	No. of examinations	No. of counts
Experimental..............	7	7	49	7	15	105
Control...................	9	7	63	9	15	135

error of the mean and the standard error of the difference of the mean.   For the purposes of this paper, a difference is considered significant when the probability of its occurrence by chance is less than 1 in 100 ($t = 2.5$, $P = 0.01$).

RESULTS
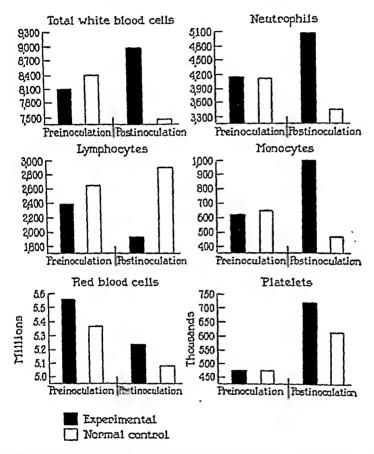
The results are indicated by the data given in Tables II and III in which are presented the mean blood cell values obtained from all counts on the experimental and control animals during the preinoculation and postinoculation periods.   Text-fig. 1 represents these values graphically.

A comparison of the mean values for the two groups in the preinoculation period revealed no significant differences.   (Red blood cells: Difference $= 180,000 \pm 132,000$; $t = 1.4$.   Platelets: Difference $= 0 \pm 42,000$.   White blood cells: Difference $= 307 \pm 366$; $t = 0.8$. Neutrophils: Difference $= 9 \pm 235$; $t = 0.04$.   Basophils: Difference $= 66 \pm 101$; $t = 0.7$.   Eosinophils: Difference $= 28 \pm 31$;

TABLE II

Mean Blood Cell Values for All Counts on Experimental and Control Groups in the Preinoculation Period

Group	Red blood cells thousands	Platelets thousands	White blood cells	Neutrophils	Basophils	Eosinophils	Lymphocytes	Monocytes
Experimental	5,554 ±88	475 ±35	8,097 ±261	4,165 ±177	754 ±78	164 ±26	2,392 ±160	623 ±48
Control	5,374 ±99	475 ±24	8,404 ±257	4,156 ±152	820 ±64	136 ±17	2,643 ±137	649 ±40

TABLE III

Mean Blood Cell Values for All Counts on Experimental and Control Groups in the Postinoculation Period

Group	Red blood cells thousands	Platelets thousands	White blood cells	Neutrophils	Basophils	Eosinophils	Lymphocytes	Monocytes
Experimental	5,245 ±48	720 ±39	8,978 ±236	5,073 ±173	821 ±47	154 ±15	1,937 ±83	996 ±47
Control	5,085 ±43	612 ±18	7,483 ±148	3,447 ±126	601 ±28	107 ±9	2,854 ±81	465 ±25

$t = 0.9$. Lymphocytes: Difference $= 251 \pm 211$; $t = 1.2$. Mono-
cytes: Difference $= 26 \pm 63$; $t = 0.4$.)

The changes in the blood cytology after inoculation are evident by
comparing the mean values for the experimental group in the postinoc-

TEXT-FIG. 1. The blood cytology in the active phase of experimental syphilis.
Mean blood cell values for all counts on the experimental and control groups in
the preinoculation and postinoculation periods.

ulation period with the mean findings for the same group in the pre-
inoculation period.   After inoculation, there was a significant increase
in the total white cell count, the platelet count, and the absolute num-
bers of neutrophils and monocytes, but the total red cell count and the

absolute number of lymphocytes were significantly decreased. No significant changes in the absolute numbers of basophils or eosinophils were noted after inoculation. (Red cell count: Difference = 297,000 ± 102,000; $t = 2.9$, $P = 0.01-$. Platelets: Difference = 245,000 ± 52,000; $t = 4.7$, $P = 0.01-$. White cell count: Difference = 881 ± 351; $t = 3.3$, $P = 0.01-$. Neutrophils: Difference = 806 ± 247; $t = 3.3$, $P = 0.01-$. Basophils: Difference = 67 ± 92; $t = 0.7$. Eosinophils: Difference = 10 ± 30; $t = 0.3$. Lymphocytes: Difference = 456 ± 180; $t = 2.5$, $P = 0.01-$. Monocytes: Difference = 373 ± 68; $t = 5.5$, $P = 0.01-$.)

A similar comparison was made between the mean values for the control group in the preinoculation and postinoculation periods. In the postinoculation period as compared with the preinoculation period, there was a slight statistically insignificant rise in the absolute number of lymphocytes and a significant increase in the platelet value. All the other blood cell elements described a change to lower levels. The mean values for the red cell count, total white cell count, and absolute numbers of neutrophils, basophils, and monocytes were significantly lower than the preinoculation mean findings. The change in the number of eosinophils was not significant. (Red blood cells: Difference = 290,000 ± 108,000; $t = 2.7$, $P = 0.01-$. Platelets: Difference = 137,000 ± 30,000; $t = 4.6$, $P = 0.01-$. White blood cells: Difference = 922 ± 297; $t = 3.1$, $P = 0.01-$. Neutrophils: Difference = 709 ± 200; $t = 3.5$, $P = 0.01-$. Basophils: Difference = 29 ± 19; $t = 1.5$. Lymphocytes: Difference = 184 ± 47; $t = 3.9$, $P = 0.01-$.)

A final comparison between the mean values for the various blood cells of the two groups in the postinoculation period revealed striking differences. The red cell count, total white cell count, platelet count, and the numbers of neutrophils, basophils, eosinophils, and monocytes of the experimental group were all significantly higher than the corresponding values for the control group, while the absolute number of lymphocytes were significantly lower. (Red blood cells: Difference = 160,000 ± 64,000; $t = 2.5$, $P = 0.01-$. Platelets: Difference = 108,000 ± 43,000; $t = 2.5$, $P = 0.01$. White blood cells: Difference = 1,495 ± 278; $t = 5.4$, $P = 0.01-$. Neutrophils: Difference

= 1,627 ± 214; t = 7.6, P = 0.01-. Basophils: Difference = 219 ± 55; t = 4.0, P = 0.01-. Eosinophils: Difference = 48 ± 17; t = 2.7, P = 0.01-. Lymphocytes: Difference = 917 ± 116; t = 7.9, P = 0.01-. Monocytes: Difference = 531 ± 53; t = 9.9, P = 0.01-.)

DISCUSSION

For the purpose of this analysis, the mean of all counts on the experimental group during the $3\frac{1}{2}$ month period after inoculation was taken to represent the findings during the active phase of the disease. In the great majority of rabbits inoculated intratesticularly with such a strain of *Tr. pallidum* as the Nichols' strain, this period covers the time of active clinical manifestations of the infection; that is, the primary orchitis, critical edema, metastatic orchitis, and generalized lesions. No attempt was made to correlate the various clinical stages of active syphilitic infection and the blood cell values during these stages. This was done in the preliminary report of Pearce previously referred to, in which the findings were charted with particular reference to the time in weeks after inoculation and to the clinical course of the disease. A future publication will discuss this matter in greater detail. The trends of the various blood cells during the $3\frac{1}{2}$ month period after inoculation may have described up and down swings, but the mean represents in general the several blood cell levels during the clinically active course of the disease since all the animals of the group presented some clinical evidence of infection at the end of the observation period.

The use of control animals counted at the same time as the experimental group served the purpose of indicating that the changes observed after inoculation may be ascribed to the disease rather than to spontaneous variations with time. Thus a change in the mean cell level of the experimental group after inoculation was considered to be significant when the following conditions were fulfilled: First, the mean value in the postinoculation period must be significantly different from the mean value in the preinoculation period, and second, the mean value in the postinoculation period must be significantly different from the mean value obtained in the control group in the postinoculation period, and furthermore, this difference must be in the same direction as observed in the first condition. These requirements were

fulfilled by the following blood components: Total white blood cells, platelets, neutrophils, lymphocytes, and monocytes. The fact that no significant differences were observed between the mean values for the experimental group and the control group in the preinoculation period indicates that the two groups were satisfactorily balanced with respect to their blood cytology before inoculation.

The differences observed in the control group of normal rabbits between the mean values for the preinoculation and postinoculation periods indicate the spontaneous variations which may occur in time. It will be noted that in the postinoculation period the values for red cells, total white cells, neutrophils, basophils, eosinophils, and monocytes were lower, and the values for lymphocytes and platelets were higher than the mean values for the preinoculation period. The preinoculation counts were made in the mid-fall of 1930 while the postinoculation period was essentially the winter of 1930–31. The changes in the cell values of the control group in the postinoculation period as compared with the preinoculation period coincide very closely with the seasonal trends of blood cells as observed in this laboratory over many years.

On the basis of the foregoing analysis, it is evident that during the $3\frac{1}{2}$ month period of disease activity, the blood cytology in experimental syphilis is characterized by an increase in the total white cell count, the platelet count, the neutrophil and monocyte counts, and a decrease in the number of lymphocytes. These changes after inoculation gain in significance when compared with the findings in the controls, since with the exception of the blood platelets, the identical blood elements of control uninoculated animals were altered in the opposite direction. It will be recalled that three animals of the original experimental group were withdrawn from consideration because they were employed for tissue studies. The inclusion of these animals in the analysis would certainly have increased the significance of the changes after inoculation since they were selected for tissue studies on the basis of the marked activity of their syphilitic lesions and the striking alterations in their blood cytology.

The change in the red blood cell level after inoculation is not definite. The mean red blood cell values of the two groups in the preinoculation period showed no statistically significant difference. In the

postinoculation period the mean value for the inoculated group was significantly lower than the preinoculation mean value. In the same time interval, however, the mean value for the control group also was significantly lower than the control preinoculation level. Thus both experimental and control groups described identical changes in time, and consequently it is probable that this change may be interpreted as a result of adaptation to environmental conditions. It is quite possible, however, that the change in the red cell count after inoculation represents an actual decrease which lacks significance in the present analysis only because of the comparatively small statistical sample. On the same basis, significant changes in the eosinophils and basophils might be demonstrated in a larger sample.

With respect to the changes in the total white cells and the neutrophils, lymphocytes, and monocytes, these findings confirm the observations of Pearce. The tables published by Bessemans and Lambin (6) indicate that their syphilitic rabbits showed a higher relative number of neutrophils and monocytes and a lower relative number of lymphocytes than the values which they obtained in a study on the blood cytology of normal rabbits. The authors, however, did not draw attention to this aspect of their investigations.

Of particular interest is the marked increase in the number of circulating monocytes during the period of disease activity. Supravital studies have demonstrated the presence of large numbers of monocytes in tissue scrapings from actively developing syphilitic lesions (4). The increased numbers of monocytes in the peripheral blood and local lesions of syphilitic rabbits indicates that they are an important participant in the cellular reaction to *Tr. pallidum*. An occasional clasmatocyte was seen in the peripheral blood, but this finding was too infrequent to be of significance in the present study.

SUMMARY

Weekly observations were made on the blood cytology of seven syphilitic and nine normal control rabbits. Each animal was examined seven times prior to and fifteen times after inoculation of the experimental group. Comparisons were made between the mean blood cell values obtained from all counts on the experimental and control groups in the preinoculation and postinoculation periods.

The mean blood cell formula of the syphilitic group for the $3\frac{1}{2}$ month period after inoculation was significantly different from the preinoculation mean values observed in the same group in the following respects: higher total white cell count, platelet count, neutrophil count, and monocyte count, and lower lymphocyte count.

The mean blood cell formula of the syphilitic group for the $3\frac{1}{2}$ month period after inoculation was significantly different from the mean blood cell formula of the normal control group in the same time interval in the following respects: higher total white cell count, platelet count, neutrophil and monocyte counts, and lower lymphocyte count.

From these results it was concluded that during the period of disease activity, the blood cytology of rabbits infected with *Tr. pallidum* is characterized by an increase in the total white cell count, the platelet, neutrophil, and monocyte counts, and a decrease in the lymphocyte count from normal values. These changes were statistically significant.

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OBSERVATIONS ON THE BLOOD CYTOLOGY IN EXPERIMENTAL SYPHILIS

II. The Period of Disease Latency

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It has been demonstrated (1, 2) that marked alterations take place in the blood cytology of syphilitic rabbits in the postinoculation period of 3 to 5 months, during which the disease is clinically characterized by the development, regression, and healing of primary and generalized lesions. Among the outstanding changes observed were an increase in the total white cell count, the platelet count, and the absolute numbers of neutrophils and monocytes, and a decrease in the number of lymphocytes. In connection with our studies on the biology of syphilitic infections, observations were made to determine the blood cell levels of infected animals after the complete spontaneous healing of lesions; that is, in the period of latency when there is no clinically demonstrable evidence of infection. It was found that the red blood cell count and the hemoglobin in per cent of rabbits with latent syphilis were both significantly lower than normal values; other significant differences were not noted. These observations, together with a brief comparison of the blood cytology in experimental and human syphilis, are presented in this report.

Material and Methods

Careful clinical examinations of a large group of syphilitic animals were made, and those rabbits selected for blood cytology studies which had been inoculated at least 5 months previously and which presented no clinical evidence of infection. Thirty-five animals from among our stock fulfilled these requirements at the time this investigation was in progress, and a study of their blood cytology forms the basis of the present report.

The 35 rabbits were all bred in this laboratory and comprised 10 hybrid crosses and 25 standard bred animals. The latter were distributed according to breed as follows: 6 Dutch, 6 Havana, 5 Himalayan, 5 English, and 3 Polish. Each

animal was housed in a separate cage and was fed a diet consisting of hay, oats, compressed food pellets, and a free supply of water.

Automatic standardized pipettes were used for the red and total white cell counts and all differential examinations were made by the neutral red supravital method, 100 cells being counted on each of two smears. The Ringer-heparin method of Casey and Helmer (3) was employed for the red cell and platelet counts, and the hemoglobin was estimated with a Newcomer hemoglobinometer.

Each animal had been inoculated with the Nichols strain of *Tr. pallidum* from 5 to 18 months (average 8 months) previous to the present study. The blood examinations of 11 of the 35 animals were conducted in April and May, 1932, and the remaining 24 animals were examined in September and October of the same year. Four complete examinations were made on each animal over a period of 2 to 4 weeks and the blood level of each animal was determined by the mean of the 4 counts. The mean and the standard error of the mean of the 35 means were then calculated for each blood element. These means were compared with normal values obtained in the following manner: In a study of the hemocytological constitution of standard bred rabbits (4), the mean blood cell levels for each of 180 normal standard bred rabbits representing 15 different pure breeds were obtained from 3 to 10 counts on each animal. The means and variance for the different cells were calculated for each breed. The means and variance of similarly obtained means for 140 miscellaneous hybrid rabbits (5) gave the normal values for hybrids. Weighted normal mean values and the standard error of these means were calculated from two formulae:

$$(1) \quad \frac{\Sigma(n_{\text{sample}} M_{\text{breed}})}{35} = Mx_{35};$$

$$(2) \quad \Sigma n_{\text{sample}} (\text{Var.breed} + M_{\text{breed}}^2) = \Sigma x_{35}^2.$$
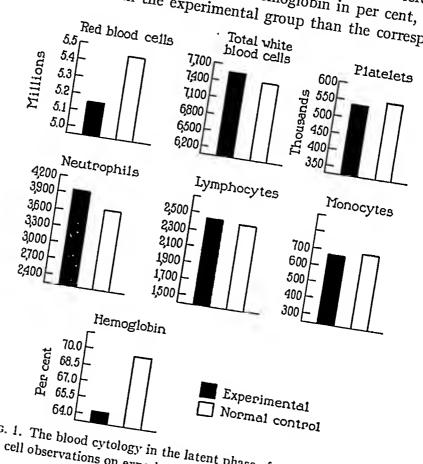
In these equations n is the number of animals of a particular breed used in the present investigation and M is the mean and Var. the variance for this breed obtained in the above mentioned study on hemocytological constitution. The value for each cell element obtained by the use of the first equation gave the weighted mean value with which the experimental mean was compared, while the standard error of these weighted means was derived by using both formulae. In all comparisons a difference was considered to be significant when the probability of its occurrence by chance was less than 1 in 100 ($t = 2.5$, $P = 0.01$).

RESULTS

Comparisons were made between the mean blood cell formula of 35 syphilitic rabbits in which clinical evidence of the disease was absent and weighted normal values obtained as described. These comparisons are presented in Table I and Text-fig. 1. There were no signifi-

TABLE I

Mean Blood Cell Values of 35 Syphilitic Rabbits with Latent Infection Compared with Normal Values

Group	Red blood cells	Platelets	White blood cells	Neutrophils	Basophils	Eosinophils	Lymphocytes	Monocytes	Hemoglobin
	thousands	*thousands*							*per cent*
Experimental......	5,154±86	537±12	7,570±236	3,942±162	415±30	85±7	2,450±129	678±53	64.6±1.0
Control...........	5,490±78	556±21	7,441±321	3,706±196	483±43	114±12	2,439±173	699±53	69.8±1.0

cant differences between the experimental and normal values with respect to the total number of white cells, the platelet count, and the absolute number of neutrophils, basophils, eosinophils, lymphocytes, or monocytes. The only significant differences encountered were in the red blood cell count and the hemoglobin in per cent, both of which were lower in the experimental group than the corresponding

TEXT-FIG. 1. The blood cytology in the latent phase of experimental syphilis. Mean blood cell observations on experimental group compared with normal control values.

normal values. (White blood cells: Difference = 129 ± 398. Platelets: Difference = 19,000 ± 21,600. Neutrophils: Difference = 236 ± 254. Basophils: Difference = 68 ± 52. Eosinophils: Difference = 29 ± 14. Lymphocytes: Difference = 11 ± 216. Monocytes: Difference = 21 ± 53. Red blood cells: Difference = 336,000 ± 116,000, t = 2.9, P = 0.01-. Hemoglobin: Difference = 5.2 ± 1.4 per cent, t = 3.6, P = 0.01-).

DISCUSSION

Experimental syphilis of the rabbit under the usual conditions of intratesticular inoculation with a strain of *Tr. pallidum* such as the Nichols' strain, is clinically manifested by the production of a primary orchitis, metastatic orchitis and generalized lesions. In our experience an orchitis of the inoculated testicle develops in all instances, while the incidence of metastatic orchitis in a group of inoculated animals varies from 80 to 100 per cent and the incidence of generalized lesions varies from 40 to 100 per cent. In a large majority of cases the tissue reaction with the production of lesions takes place within a 2 to 3 month period following inoculation after which there is a regression of all lesions and finally complete healing. With the appearance of this stage there is established a period of latency so far as clinical evidence of the disease is concerned, but the animal still harbors infective spirochetes as can be demonstrated by subinoculation of lymph node tissue. In a certain proportion of animals, the period of latency is interrupted by one or more periods of clinical relapse with the production of lesions. Ultimately, however, a state of permanent latency is reached in the vast majority of animals.

All the animals of the present experiment had passed through the period of lesion activity, and at the time of the blood studies the disease in all was latent. A primary orchitis had developed in all animals, and the disease in the group can be characterized as of moderate severity varying in individual animals from a very mild disease with no generalized lesions to a very severe infection with many generalized lesions.

Previous studies (1, 2) have demonstrated that the active period of experimental syphilis of the rabbit is associated with changes in the blood cytology which include an increase in the total white cell count, platelet count, and numbers of neutrophils and monocytes, and a decrease in the number of lymphocytes. These changes were shown to be of statistical significance. From the present study it is seen that when the period of latency has been established, these blood cell elements return to normal levels but the red blood cells and hemoglobin are significantly depressed. The depression of the red cells is of increased significance since the blood examinations were conducted in April and May, September and October. Observations on the sea-

sonal trends of blood cells made in this laboratory over several years has indicated that the red cell count is highest during these months. From a study of the blood cytology in untreated and treated human syphilis (6), it was found that with treatment there is a fall in the total white cell count, the platelet count, and the numbers of neutrophils and monocytes, and a rise in the number of lymphocytes from values observed in untreated cases. These findings are noteworthy since they are paralleled so closely by the findings in the experimental disease. The blood cell values of syphilitic rabbits in the active phase of the disease differed from normal values in the following respects: higher white blood cell count, platelet count, neutrophil and monocyte counts, and lower lymphocyte count. In the present study it has been shown that these cells return to normal levels during the period of latency. With respect to these elements, therefore, the changes observed in the experimental disease after spontaneous healing of all lesions are similar in direction to the blood cell changes in human syphilis following the institution of treatment. The depression of the red cells and hemoglobin after complete healing of lesions in the rabbit has its counterpart also in the human disease. In untreated tertiary human syphilis, the red cells and hemoglobin were significantly lower than the values for treated tertiary syphilis patients, while no differences were found between any of the other blood cells of the two groups. Thus a comparison of the blood formula of syphilitic rabbits in the period of latency with normal control values revealed the same differences as were noted when comparing the blood formula of untreated with treated tertiary syphilis in man. It is quite probable that treatment in the latent period of experimental syphilis would bring about an increase in the red cell count and hemoglobin just as it does in the tertiary disease of man.

SUMMARY

The mean blood cell levels of 35 latent syphilitic rabbits in which all lesions had undergone spontaneous regression and complete healing were compared with weighted values for normal rabbits. The only differences noted were in the red cell count and hemoglobin content, both of which were significantly lower in the experimental group than the normal values.

A parallelism was observed between the blood cell changes of the experimental disease after spontaneous regression of lesions, and the cell changes in the human disease after treatment. This parallelism lends additional weight to deductions drawn from the experimental disease as applied to human syphilis.

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STUDIES ON PSEUDORABIES (INFECTIOUS BULBAR PARALYSIS, MAD ITCH)

II. Routes of Infection in the Rabbit, with Remarks on the Relation of the Virus to Other Viruses Affecting the Nervous System

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In a previous communication (Hurst, 1933) the histology of pseudorabies in the rabbit has been considered. While clearly possessed of neurotropic affinities, in this animal the virus does not behave as a strict neurotrope, but produces intranuclear inclusions in cells derived from any embryonic layer. It is interesting now to compare, from the experimental standpoint, the manner of spread of this virus with that of the more purely neurotropic viruses (poliomyelitis, rabies, Borna).

Technique

The incubation periods in four rabbits inoculated subcutaneously with 1 cc. of the supernatant fluid from a 10 per cent unglycerinated brain suspension lasted 51, 52, 54 and 57 hours respectively. With amounts of suspension near the minimal infecting dose, or with very old glycerinated material, the incubation period may be 8 days or longer. In all the passage experiments listed in the following tables fresh material was employed, and a long incubation period was taken as indicating the presence of a minimal amount of virus; except where otherwise stated 1 cc. of a 10 per cent suspension constituted the test dose.

The minimal infecting dose of the Iowa strain of virus was determined for rabbits by Shope (1933) to be not greater than 0.01 mg. wet brain for intracerebral inoculation, and 0.1 mg. for subcutaneous inoculation. Recent tests indicate that the latter dose lies between 0.1 and 0.01 mg., and is often nearer the second figure. Where exact amounts of virus are mentioned in the ensuing experiments the inoculum was titrated for potency at the time of use and the quantities are stated in terms of the minimal dose for subcutaneous inoculation. Dr. M. H. Merrill has found the minimal subcutaneous infecting dose of the Aujeszky virus employed to lie between 0.05 and 0.025 mg. wet brain.

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STUDIES ON PSEUDORABIES. II

EXPERIMENTAL RESULTS

Evidence of Neural Transmission of the Virus

Iowa Strain.—In infections following subcutaneous, intradermal or intramuscular inoculation (Table I), virus may sometimes be detected in the defibrinated blood provided that sufficient quantity be tested.

Injecting several small samples of blood, Shope (1931) was unable to detect the presence of virus; it is now obvious that even in relatively enormous quantities virus is inconstantly found. Its absence from

TABLE I

Distribution of Iowa Virus Following Intramuscular, Subcutaneous or Intradermal Inoculation into Rabbits

No.	Inoculated	First symptom	Killed or died	Presence of virus in							
				Cerebral cortex	Cervical cord	Dorsal cord	Lumbar cord	Sciatic nerve	Lung	Spleen	Defibrinated blood (6–10 cc.)
		hrs.	*hrs.*								
1	Intramuscular, leg	50	K 50	0	—	—	+72	+144	+90	+120	+124
2	Subcutaneous, flank	52	D 72	+120	—	+52	—	—	—	—	+124
3	Subcutaneous, leg	68	K 74	0	+116	—	+52	+54	+140	0	0
4	Intradermal, leg	?	D 96	0	0	+122	+55	+54	0	—	—
5	Subcutaneous, flank	190	K 190	0	+73	+50	+120	—	—	—	—

+ = development of mad itch with incubation period in hours. − = not tested. 0 = no take.

the blood constitutes the chief point of distinction between it and the classical Aujeszky virus, which is easily detected in minute amounts of blood or serum (Table VI).

In view of its scanty virus content, in the case of positive inoculations with various organs the contained blood cannot be held responsible; small amounts of virus are inconstantly detected in the lungs and spleen, sometimes in its apparent absence from the blood (R 3).

Shope found virus in the liver in one of three cases.

By sacrificing the animals at the appropriate time, close relation can be demonstrated between the position of the inoculated area

and the virus content of various parts of the central nervous system. After injection into the flank, abundant virus may exist at the corresponding level of the spinal cord, less in the cervical and lumbar cords (R 5) and little or none in the cerebral cortex (R 2 and 5). With inoculation in the leg, virus is present in greatest quantity in the sciatic nerve and lumbar cord (R 1, 3, 4), and in diminishing amounts at higher levels. As already recorded (1933), death occurs with a comparatively low concentration of virus in the medullary centers. While in the cord the differential distribution of virus is, of course, most evident when itching first begins, *i.e.* soon after the virus has reached the spinal ganglia and segments corresponding to the local

TABLE II

Distribution of Iowa Virus Following Intracerebral Inoculation into Rabbits

No.	Killed or died	Symptoms	Presence of virus in							
			Cerebral cortex	Gasserian ganglion	Cervical cord	Lumbar cord	Sciatic nerve	Lung	Spleen	Defibrinated blood (6–10 cc.)
	hrs.									
8	K 34	None	+50	+60	+60	+70	0	0	+90	+70
9	D 46	Typical—1 hr.	+52	+66	+53	+73	0	+120	+120	0
10	D 92	Typical—4 hrs.	+52	—	+72	+144	0	+88	0	0

lesion, the cerebral cortex may still be uninfected at death (R 4). These findings forcibly suggest an ascending infection by the nervous pathway.

After intracerebral inoculation (Table II) virus is again present inconstantly and in small amount in the blood, spleen and lungs. Diminishing quantities down the nervous axis and absence from the sciatic nerve reverse the distribution indicated in Table I. It is now evident that the sterility of the cerebral cortex in the preceding experiments does not betoken insusceptibility of this part of the nervous system to the action of the virus.

Further evidence in favor of spread by the nervous route is afforded in Tables III and IV. The salivary glands are often infective after intracerebral inoculation or after subcutaneous inoculation into the

base of the ear, and rarely so if injection is practised subcutaneously in the flank or foot. The adrenals are frequently infective after subcutaneous inoculation into the flank, but not after injection into

TABLE III

Infectivity of Adrenal and Salivary Glands at Death Following Inoculation of Iowa Virus at Various Sites in Rabbits

Route of inoculation	No. of animals	Presence of virus in	
		Adrenal	Salivary
Subcutaneous, base of ear.........	5	0, 0, 0, +83, 0	+70, +86, 0, +68, 0
Subcutaneous, flank.............	8	+66, +180, —, —, +68, +82, +65, 0	0, 0, 0, 0, +98, 0, —, —
Subcutaneous, dorsum of foot......	4	0, 0, 0, 0	0, 0, 0, 0
Intracerebral...............	4	0, 0, +106, 0	+144, +85, +122, 0

TABLE IV

Duration of Incubation Period Following Subcutaneous Inoculation at Various Sites of Iowa Virus into Rabbits

Dose of virus in M.I.D.	Inoculation into		
	Base of ear	Dorsum of foot	Dorsum of foot after removal of sciatic nerve
5,000	+58, +62, +68	+68, +71, +72	+75, +86, +89
25	+85	+92, +115	+122, +144, +161
100	+62		Removal of sciatic and femoral nerves
250	+55	+70	+102, +126
		+65	+80, +86

Four different samples of virus were used in these experiments, but the data in each horizontal row were obtained with one sample and on one occasion.

the leg or ear. If we admit that spread occurs by whatever nervous paths are available, these observations are readily explainable. In a disease of superlatively rapid progression there might be time for virus

E. WESTON HURST

infecting primarily, say, the dorsal cord, to migrate to the adrenals, whereas arriving soon before death in the brain stem it would not have time to pass out to the salivary glands. Conversely, virus reaching the brain stem from the ear would infect the salivary glands but not the adrenals. In the case of primary lumbar infection neither would be affected. Again, as shown in the left hand columns of Table IV, the

TABLE V

Distribution of Iowa Virus during Incubation Period Following Intradermal, Subcutaneous or Intramuscular Inoculation into Rabbits

No.	Killed	Route of inoculation	Amount of 5 per cent suspension	Presence of virus in		
				Site of inoculation	Blood (10–13 cc.)	Spinal cord and ganglia
	min.		cc.			
11	30	Subcutaneous	5	—	0*	—
12	90	Subcutaneous	5	—	0*	—
	hrs.				0†	
13	6	Intramuscular	2	—	0*	0
14–15	12	Intradermal	1	+84, +96	0*	0
16–17	12	Intradermal	3	—	0†	0
18	16	Intramuscular	1	+53	0*	0
19–20	18	Intradermal	1	+60, +120	0*	0
21–22	24	Intradermal	1	+50, +66	0†	0
23	28	Intramuscular	1	+144	0*	0
24	36	Intradermal	1	+60	0†	0
25	40	Intramuscular	1	+64	0*	+82
26	48	Intradermal	1	+62	0*	+72
27	52	Intramuscular	1	+54	+210†	

* Inoculation of defibrinated blood.
† Direct inoculation of whole blood.
Controls developed symptoms at 49 and 53 hours (intradermal) and 55 hours (intramuscular) respectively.

duration of the incubation period following subcutaneous inoculation increases materially with greater length of the nervous pathway. In another experiment a single animal was inoculated at the same moment in the ear, the flank and the foot. An interval of several hours separated the onset of itching in the ear and flank, affected in this order, while the rabbit died before itching began in the foot; here the intervention of individual variations in susceptibility was fully excluded.

The data presented in Table V also apparently rule out the possibility of blood transmission, though later experiments will show that these results cannot be accepted at their face value. Even with considerable volumes of inoculum (over 12,000 M.I.D.) no transient leakage into the blood stream is detectable during the first hour or two, and apparent sterility as concerns the virus is maintained to almost the end of the incubation period. To be more precise, the experiments indicate the presence at one time in the whole circulating fluid of less than about 10 M.I.D. virus. The spinal cord and ganglia may show virus before the blood, while at the site of inoculation active increase is occurring.

TABLE VI

Distribution of Aujeszky Virus Following Subcutaneous Inoculation into Rabbits

No.	Inoculated	First symptom	Killed or died	Presence of virus in										
				Cerebral cortex	Cervical cord	Dorsal cord	Lumbar cord	Sciatic nerve	Lung	Spleen	Adrenal	Salivary	Defibrinated blood	
													0.25 cc.	5.0 cc.
		hrs.	hrs.											
70	Flank	—	K 30	0	0	+72	—	—	+84	+110	—	—	0	+149
71	Flank	—	K 33	0	0	+96	—	—	+120	+140	—	—	0	+180
72	Flank	—	K 42	0	0	+86	—	—	+80	+86	—	—	+86	+72
6	Flank	47	K 48	+78	—	+66	—	—	+47	+47	+49	+66	+47	+49
7	Leg	46	D 53	+65	—	—	+60	+48	+45	+48	+50	+48	+74	+74

Finally, the histological findings already reported (1933) point also to nervous transmission of the virus. It seems, therefore, that indubitable evidence of spread of the Iowa virus by the nervous route is forthcoming.

Aujeszky Strain.—Similar study of the course of infection with the Aujeszky virus (Table VI) is complicated by the infectivity, towards the end, of even minute amounts of blood, rendering difficult the correct evaluation of positive inoculations with the various viscera. If, however, examination is made sufficiently early, the blood is infective only in large doses, and the recovery of virus from lung and spleen can be accepted as significant. Experiment showed

that using 5 cc. as the test inoculum the blood is uninfective at 1/2, 6 and 12 hours after subcutaneous inoculation; from about 24 hours onwards uniformly positive results are obtained. The detection of virus, at a rather later stage, in only that part of the nervous system directly connected with the site of inoculation (R 70–72) suggests that, as is the case with the Iowa strain, nervous infection is brought about by the neural route, and not, as previous observers (Schmiedhoffer, 1910; Remlinger and Bailly, 1933) have held, by the circulation. This conclusion is supported by the histological findings. It will be noted that the virus appears in the cord at a much earlier period than does the Iowa strain.

Effect of Nerve Section on the Course of Infection Following Subcutaneous Inoculation

If in the case of the Iowa virus introduced subcutaneously spread is possible only by the nervous route, ablation of the nerve supply beforehand should prevent infection. Bertarelli and Melli (1913) claimed that in some cases tying or removal of a part of the nerve protected against inoculation of the peripheral segment.

In the present investigation six rabbits underwent removal of over an inch of the sciatic nerve of one side, and four of the sciatic and femoral nerves of one thigh. Ether was the anesthetic employed. Immediately after closure of the wound (sealed with collodion), or after an interval of 3 weeks from the date of excision, virus was inoculated subcutaneously in the dorsum of the denervated foot. In all cases a lengthened incubation period was noted, but finally every animal died (Table IV). One scratched the site of operation in the thigh, four scratched areas unrelated to the excised nerve (opposite flank or opposite hind limb) and five died suddenly without having scratched. Three of the last group were examined for the presence of virus in the cord and medulla, with positive results; virus was also present in the lungs, spleen and blood.

The conclusion seems unavoidable that in the absence of nervous connections the Iowa virus may reach the nervous system through the medium of the blood. The following experiments reveal, however, the interesting fact that this spread is not direct.

Results of Intravenous Inoculation of Virus

Infection regularly ensues after intravenous introduction of even small doses of virus; in contrast to the figures for most neurotropic viruses (poliomyelitis, rabies, etc.), in the present instance the minimal infecting dose is only 3–10 times that for subcutaneous inoculation. The incubation period is a few hours longer than after subcutaneous or intramuscular injection of the same quantity into the flank. The symptoms vary. Rather less than half the animals die suddenly, or exhibit only some restlessness with rapid shallow breathing before exitus. The remainder itch; in each of thirteen cases this itching occurred on some part of the trunk or limbs (thrice on a fore limb, twice on a hind limb, eight times on one or both flanks and never on the head).

In addition to the lesions previously described as occurring after peripheral or intracerebral inoculation, the adrenals of rabbits receiving large doses of virus show, with fair regularity, considerable areas of acute necrosis in both cortex and medulla; these closely resemble the similar areas in herpes (Smith, 1931). Hemorrhage and infiltration with polymorphonuclear leucocytes and a few eosinophils accompany the destruction. Acidophilic nuclear inclusions, more coarsely granular than those in the nervous tissues and very like those of herpes, exist in cells surrounding the necrotic areas, while the sympathetic ganglia on the surface of the glands show typical alterations in nerve cells, capsule cells and neurilemmal nuclei.

When itching occurs the corresponding spinal ganglia are typically affected, but, as with subcutaneous inoculation, at death no definite lesions are discernible in the cerebral cortex or brain stem.

The progress of infection as revealed by experimental study is as follows:

The distribution of the Iowa strain after the administration of massive doses is set forth in Table VII. At the end of 1/2 hour a small quantity of virus is still present in the blood, but after 2 hours all, or almost all, has disappeared. Using the usual test dose of 1 cc. of a 10 per cent emulsion, no virus is now detectable in any of the viscera. In the belief that this result could not represent the true state of affairs, another rabbit was given 5000 M.I.D. (i.e. roughly 3 M.I.D. per gm. body tissues) and larger amounts of the various organs emulsified; where possible, doses of 1, 5 and 20 cc. of the supernatant fluid

TABLE VII

Distribution of Iowa Virus Following Intravenous Inoculation of Massive Doses into Rabbits

No.	Dose in M.L.D.	First symptom	Killed or died	Presence of virus in											
				Cerebral cortex	Medulla	Cervical cord	Dorsal cord	Lumbar cord	Lung	Spleen	Liver	Kidney	Adrenal	Testis or ovary	Defibrinated blood (10–12 cc.)
		hrs.	hrs.												
53	5,000	—	1/2	—	—	—	—	—	—	—	—	—	—	—	+133
54	5,000	—	2	—	—	—	—	—	o	o	o	o	0	0 (O)	0
55	10,000	—	2	—	—	—	—	—	o	o	o	—	—	—	+232
56	2,500	—	16	o	o	o	o	o	0	o	o	+140	+70	0 (T)	0
57	2,500	—	24	o	o	o	o	o	+110	o	o	—	—	—	+165
58	2,500	—	24	o	o	o	+130	o	+79	o	o	+72	+72	+64 (T)	+117
59	2,500	—	40	o	o	o	o	o	+116	o	o	—	—	—	+85
60	2,500	—	48	o	o	+122	+92	+113	+60	+101	o	—	—	—	+90
61	2,500	—	48	+91	+69	—	—	+76	+79	+135	o	—	—	—	+72
62	2,500	58*	58	o	+182	—	—	+69	+72	+91	+120	+72	+80	+100 (T)	+108
29	1,000	64†	72	o	+72	—	—	+75	+88	+130	0	+126	+59	—	—
31	1,000	?*	90	o	+72	—	+130	+126	+135	0	—	—	+91	—	—

* Did not itch.

† Bit and scratched.

from a 10 per cent suspension were injected into fresh animals. Positive results were now obtained with the lung, spleen, kidneys and ovaries, and negative with the blood, urine, liver, adrenals, brain and cord, and mixed subcutaneous and muscular tissues. By comparing the weights of the organs and skeletal tissues with the results obtained the following quantitative distribution of virus was determined. Lung > 22 < 110 M.I.D., spleen > $2\frac{1}{2}$ < 12 M.I.D., kidneys > 25 < 125 M.I.D., ovaries > 2 < 6 M.I.D., blood < 6 M.I.D., liver < 40 M.I.D., adrenals < 2 M.I.D., brain and cord < 6 M.I.D., skeletal tissues < 600 M.I.D. The length of the incubation periods in the passage animals indicated that the actual figures were probably much less than the maxima suggested. When, to eliminate the possibility of virus being already firmly bound to the cellular elements of the viscera, two experiments were performed, similar save for the fact that whole emulsions were substituted for the supernatant fluids, definitely lower figures were obtained. This is probably an example of the inhibitory effect exercised by thick tissue suspension on the action of a virus, a phenomenon already known in the case of poliomyelitis and other viruses. At the most generous estimate, therefore, fourfifths of the virus introduced could not be recovered within 2 hours of injection. Even if the higher limits indicated for the virus content of the lung, spleen and kidney were the correct ones, they represented in these organs only an approximately threefold concentration over what should obtain with uniform distribution; in the case of the ovaries the concentration was possibly sixfold.

This loss of virus may possibly be due to inactivation or destruction by the elements of the blood, for titration of a mixture of proportional quantities of virus and freshly drawn heparinized or defibrinated blood, incubated for 2 hours at 37°C., showed roughly a 90 per cent decrease in activity. This observation makes it very unlikely that pseudorabies virus multiplies in the blood.

With the usual test doses, from 24 hours onwards virus is found constantly in the lungs, kidney and adrenals. The spleen is usually infective towards the end of the incubation period and during the symptomatic stage. At a similar stage virus is present in the testis, but only inconstantly in the liver. Virus returns to the blood within 24 hours, and thereafter persists in the circulation; this is in contrast

to the sterility obtaining for a much longer time after subcutaneous inoculation.

Virus is usually absent from the nervous system until late in the incubation period. In three rabbits killed in the presymptomatic stage (R 59, 60, 61) the cord was infective before the medulla or cerebral cortex. In two during the period of the developed disease (R 62, 31) the infectivity of the medulla was equal to or greater than that of the cord; these animals did not itch. In one case (R 29) the reverse obtained; this animal itched. In two fatal cases (R 29, 31) the cerebral cortex was still uninfected at death.

This irregular distribution of virus throughout the nervous system does not suggest infection directly from the blood. It has already been seen that at the 2nd hour no virus has, apparently, settled in the nervous tissues. Moreover, if infection of the nervous system occurred at the time of introduction of the virus, in addition to the lesions constantly found after intracerebral inoculation we should expect a shorter incubation period, since minute amounts of virus must be inoculated intracerebrally to give an incubation period of over 50 hours. If longer incubation were attributable to delay in passage of virus through, say, the capillary walls, lesions in the endothelial cells might be expected; the absence of any lesions in the brain or brain stem has already received comment.

On the other hand, if from the foci of infection obviously established in various viscera during the first few minutes after inoculation virus were to spread by the nervous route, the findings are readily explainable. In the majority of cases, owing to proximity to the viscera, the spinal cord and ganglia would be first infected, lesions produced here and referred itching evoked. Thus we might explain the observations in the case of R 29. In other cases spread to the medulla might occur (perhaps from the lungs) by the vagus; if this were sufficiently rapid the animal might die of medullary paralysis before the cord and ganglia were sufficiently affected to give rise to peripheral irritation (e.g., as in R 62 and 31). That the lesions observed in the spinal ganglia of itching animals result from the ascent of virus deposited in the subcutaneous or muscular tissues is unlikely for several reasons. In the first place no considerable quantity can be detected here at the 2nd hour; amounts so minute as to escape detection would be incapable

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of producing the disease with the length of incubation noted in the experiments. Secondly it would be anticipated that a certain number of animals would itch on the head or neck, whereas in each of thirteen instances itching was confined to the trunk or limbs. Thirdly, in two of five cases the area bitten contained no detectable amount of virus; in the other three the incubation periods in the passage animals were 73, 93 and 204 hours respectively. These figures contrast with those

TABLE VIII

Distribution of Iowa Virus Following Intravenous Inoculation of Small Doses into Rabbits

No.	Dose in M.I.D.	First symptoms	Killed	Presence of virus in											
				Cerebral cortex	Medulla	Cervical cord	Dorsal cord	Lumbar cord	Lung	Spleen	Liver	Kidney	Adrenal	Testis or ovary	Defibrinated blood (10–12 cc.)
63	60	—	24	—											
64	60	—	36	—	—	0	0	0							
65	60	—	48	—	0	0	0	0	0	0	0				
66	60	—	60	0	0	0	0	0	+124	0	0	0	0	0 (T)	0
67	60	—	64	0	0	0	0	0	0	0	0	0	0	0 (T)	0
68	20	—	66	0	0	0	0	+108	0	0	0	+69	+133	0 (O)	0
69	60	70*	71	0	—	+87	+120	+122	0	0	0	+65	+87	0 (T)	+120

Controls for Nos. 63–66 and 69: Subcutaneous +68; intravenous +71.
Control for No. 67: Intravenous +64.
Controls for No. 68: Subcutaneous +64; intravenous +74.
* Bit and scratched.

for the local lesion at a corresponding period after subcutaneous or intramuscular inoculation (Table V); they would probably be in keeping with the presence of a small amount of virus passed centrifugally from a central nervous reservoir. Finally, three rabbits were inoculated, one directly into the kidney, one into the liver and one into the lung; before death each scratched the flank or shoulder. (The organ inoculated showed in each case acute necrotic lesions with abundant nuclear inclusions in the cells characteristic of the particular organ; i.e., in cells derived from mesoderm and entoderm.) It would

seem, therefore, that itching after intravenous inoculation is a referred sensation from a central lesion determined by virus originating from some source other than the area scratched.

If a much smaller dose be given intravenously (Table VIII) its distribution during the incubation period, though showing similarities to that in the preceding table, is more erratic. It is to be noted that the condition of the blood more nearly resembles that after subcutaneous inoculation in rarely containing detectable virus.

The distribution of the Aujeszky strain after intravenous inoculation was not fully worked out; it was determined, however, that 2 hours after administration of 5000 M.I.D. the blood in doses of 1, 5 and 20 cc. did not reproduce the disease in passage animals (*i.e.*, contained < 6 M.I.D. virus).

An important fact emerging from these experiments by the intravenous route is that if after subcutaneous inoculation virus were to enter the circulation intermittently, even in considerable amount, its rapid disappearance therefrom would lead to the restitution of sterility. Thus the results charted in Table V are not in themselves sufficient to eliminate the possibility of blood spread; on the other hand the presence of virus in the lung and spleen in its absence from the blood (Tables I and II), and the possibility of infection resulting from inoculation in a denervated area are readily explained. Again, it seems that when virus is detectable in the blood at the end of the incubation period or earlier, according to experimental conditions and virus used, its presence here must be due to one or both of two factors, its continuous emission in considerable amount from the primary or other focus, or failure of the mechanism for its removal.

Appearance of Virus in the Blood after Subcutaneous Inoculation

The possibility that the appearance of virus in the blood is due to failure of some absorptive mechanism is excluded by the following experiment. Two animals were inoculated subcutaneously with Aujeszky virus. At the time when virus was expected to appear in the blood stream (22 and 26 hours), a test bleeding was performed and various amounts of blood inoculated into fresh animals. The original pair of animals was then inoculated intravenously with 5000 M.I.D. virus; 2 hours later they were killed and the blood was titrated

once more. In neither animal was its virus content appreciably higher than before.

Acting on the assumption that the reticulo-endothelial system might be concerned in the removal of virus from the circulation, an attempt was made to demonstrate the Iowa strain in the blood stream at various stages of the incubation period in animals previously treated with India ink.

Higgin's non-waterproof American Drawing Ink was dialyzed against sterile Locke's solution changed each alternate day for 10 days. Doses of 5 cc. ink diluted to 7.5 cc. with sterile Locke's solution were injected intravenously into rabbits on each of 2 successive days. The animals were then subjected to subcutaneous inoculation with the Iowa virus, and the ink injections continued daily. At various periods the rabbits were killed and the heart blood titrated in fresh animals.

About a year had elapsed since the experiments listed in Table V were performed, in which time the virus had been passed on a number of occasions. Control rabbits not receiving India ink now frequently showed small quantities of virus in the blood during the incubation period, and the differences in this respect between them and the animals given ink were not sufficiently marked to be considered significant. These observations probably indicate that with passage the Iowa virus is approximating to the Aujeszky strain in its capacity for invading the blood stream.

Existing records do not, unfortunately, furnish precise information regarding the ease with which newly isolated strains of the Aujeszky virus invaded the blood stream. The following statements seem to indicate that different strains, or the same strain at different times, may vary in this respect. Aujeszky (1902) stated that the virus was almost always in the blood, thus implying a certain number of negative tests; in our experience the Aujeszky virus now used is always present in, and may be detected in minute amounts of the blood of infected rabbits. Isabolinsky and Patzewitsch (1912) found the blood infective only in large doses and after a long incubation period. Sangiorgi (1914) could detect virus in the blood only just before death. It is possible that with repeated passage the Iowa virus may appear in the blood stream in larger amount than heretofore, in which case the only distinguishing feature between it and the Aujeszky strain in common use will disappear and complete uniformity of behavior be established between the two viruses.

DISCUSSION

The more strictly neurotropic viruses (poliomyelitis, rabies, Borna) reach the central nervous system from the periphery by the neural route, and there induce a primary degeneration of nerve cells accompanied or followed, as the case may be, by reactive phenomena in the vascular and interstitial tissues. The evidence presented in this and in a previous paper shows that the same is true of the pseudorabies virus, which is clearly neurotropic. In the rabbit death ensues before the primary attack on the neurons is masked by reactive change. But whereas viruses of the former group are rarely or never detected in the blood stream, if at all only during the period when wholesale destruction and phagocytosis of nerve cells may account for some leakage into the circulation, the capacity of the Aujeszky virus for abundant increase in non-nervous tissue enables it to establish a primary extraneural focus, from which, apparently, considerable quantities of virus are emitted into the blood from an early stage of the incubation period. The differences observed in this respect between the Aujeszky and the Iowa strain indicate that the circulation of virus in the prenervous phase of the disease is, unfortunately, an occurrence insufficiently constant to serve as a means of distinguishing this type of virus from the stricter neurotropes. Moderate amounts of virus can be dealt with by a mechanism of removal leaving the blood free from detectable contamination. Under these circumstances it is even uncertain what the real position regarding the stricter neurotropes may be, except that as far as is known they do not multiply to any extent outside the nervous system.

Of greater value in differentiating between the two types of virus is the observation that while the stricter neurotropes just mentioned, when introduced into the blood stream in any amount less than an overwhelming one, do not cause infection, the minimal intravenous infecting dose of pseudorabies virus is but little greater than that for subcutaneous or intramuscular inoculation. It is important to recognize that this property of the pseudorabies virus is not due to ability to penetrate directly the hemato-encephalic barrier; rather its pluricellular affinities, abundantly evident on histological study, enable it to establish multiple infective foci from which follows extension by the

neural route. Except when they are of the nature of a general response to infection, the lesions of rabies and Borna disease in organs other than the nervous system occur primarily in connection with the nervous apparatus of the organ, which they reach by centrifugal spread along the nerve trunks. In the case of pseudorabies the results of direct inoculation into a given organ (liver, lung or kidney), together with numerous other observations, clearly demonstrate the capacity of the virus for evoking primary and specific lesions in cells derived from entoderm and mesoderm as well as in those of ectodermal origin.

In many ways remarkable similarity exists between the pseudorabies and the herpes virus. Goodpasture and Teague (1923 a, 1925 a), Smith (1931) and others have called attention to the pluricellular affinities of the latter. The herpes virus does not appear in the blood (Goodpasture, 1925 b), or its presence there is ephemeral during the acute stages of the malady (Levaditi, 1926). Hence no doubt the negative results of intramuscular inoculation following nerve section (Goodpasture and Teague, 1923 b; Levaditi, 1926). From our experience with pseudorabies we can, however, envisage the possibility of herpes virus introduced into a denervated area growing locally and entering the blood stream undetected, but, provided that its affinity for the general viscera were less than is the case with the pseudorabies virus, of its frequent failure in low concentration to establish infective visceral foci from which spread to the nervous system might follow. Occasional success in this direction might account for the results of Marinesco and Draganesco (1932) which, they assume, discount Goodpasture's evidence for axonal spread of the virus (1925 b). While a few workers (le Fèvre de Arric and Millet, 1925; Levaditi, 1926) have found intravenous inoculation unattended by cerebral trauma rarely effective in inducing encephalitis, many observers (Doerr and Vöchting, 1920; Luger and Lauda, 1921; Remlinger and Bailly, 1925; Goodpasture and Teague, 1923 b) seem to have encountered little difficulty in producing infection by this route. The lesions in the adrenals following intravenous injection of herpes virus (Smith, 1931) are similar to those described in the present paper. In all the cases quoted the dosage has been fairly large, but far from comparable with that requisite in the case of poliomyelitis or rabies. Although Good-

pasture and Teague felt that their success with intravenous inoculation did not warrant the categorical assertion that the axis cylinders are the only portal of entry of the virus to the central nervous system, it seems highly probable that an *ad hoc* inquiry would reveal after intravenous inoculation of herpetic material a state of affairs similar to that obtaining in pseudorabies; *viz.*, an ascending neural infection from primary foci set up in various viscera.

At first sight the behavior of certain other neurotropic viruses appears different from that of those thus far dealt with. In horses dying of equine encephalomyelitis[1] animal inoculation demonstrates the existence of virus only in the central nervous system, in which the lesions are typical of an acute neurotropic virus disease. Before nervous symptoms are manifest, however, virus may be present in the blood in considerable quantities. Much the same is true of the guinea pig (Howitt, 1932), in which virus may early be recovered from the blood and viscera, but later only from the nervous system and perhaps the salivary glands and adrenals. That the organism is evidently capable of multiplication in situations outside the nervous tissues follows also from the work of Syverton, Cox and Olitsky (1933).

Almost identical findings are recorded in the case of the neurotropic modification of the yellow fever virus. At death in the mouse (Theiler, 1930; Dinger, 1931), guinea pig (Lloyd, Penna and Mahaffy, 1933; Theiler, 1933) and monkey (Lloyd and Penna, 1933) virus appears to be localized in the central and peripheral nervous system, adrenals and perhaps salivary glands, leaving the blood and other viscera free. The circulation of virus in the blood, immediately following massive intraperitoneal inoculation, may no doubt legitimately be compared with the absorption of a non-specific agent such as ovalbumin (Hughes and Theiler, 1934). But Sawyer and Lloyd (1931) found the blood infective for 4 and possibly 5 days, while after administering minute doses Hughes and Theiler noted a latent period preceding the appearance of virus, which then circulated for from 1–3 days; in the intervening period multiplication must have taken place.

Webster and Fite state[2] that louping ill virus introduced intra-

[1] Work shortly to be published.
[2] Webster, L. T., and Fite, G. L., personal communication.

nasally, and presumably entering the body in comparatively small amounts, may be detected in appreciable quantities in the blood of mice during the prenervous phase, though when nervous symptoms develop it is absent therefrom. Pool, Brownlee and Wilson (1930), Gordon *et al.* (1932) and MacLeod and Gordon (1932) have already shown that in the natural host, the sheep, the blood is infective during the febrile stages of the disease, that such infectivity is not necessarily followed by the development of nervous symptoms, that the contagium is transmissible by a blood-sucking insect (*Ixodes ricinus* L.), and that intravenous inoculation of virus may be effective in producing the typical disease.

If this third group of neurotropic viruses be regarded as having affinities for tissues other than the nervous system, and at the same time a slower rate of progression along the nerves than the pseudorabies virus, it is possible to imagine that the systemic and nervous phases of the diseases they cause may become temporally dissociated. The visceral reaction occurs and the virus is overcome. But virus which has meanwhile travelled centripetally along the nerves now finds itself in a susceptible tissue isolated by the hemato-encephalic barrier from humoral influences. Encephalitis follows. If this interpretation of the facts is correct, the viruses of this group are distinguishable from that of pseudorabies chiefly by the accident of their slower migration along the nerves.

In the matter of nomenclature we may perhaps refer to these viruses with pluricellular affinities as pantropic. The chief objection to designating them viscerotropic and neurotropic viruses lies in the possible implication that the factors making for general tissue affinity on the one hand and neurotropism on the other are separable and independent, being analogous in this respect to the flagellar and somatic antigens of bacteria. Though of course this may prove to be the case, none of the facts at present ascertained necessitates the assumption. The acquisition of more pronounced neurotropism on brain-to-brain transfer may well indicate a basic alteration in the virus as an entity, impressed on it by the medium of culture; the dissociation of visceral and nervous manifestations of virus action to a slow progression along the nerves coupled with the peculiar physiological semi-isolation of the nervous system.

Levaditi's conception of a group of viruses linked by a selective affinity for skin and nervous tissue is considered by many workers to be based on incomplete observation and undue elasticity in interpretation of fact. Thus poliomyelitis and rabies viruses have no particular affinity for the dermis; herpes virus attacks cells derived from all three embryonic layers; the lesions of cerebral vaccinia are largely meningeal or ascribable to meningeal lesions. If a classification of viruses in terms of their cellular affinities were desired, a primary grouping according as to whether they are neurotropic, viscerotropic or pantropic might supply the framework The supposed characteristics of the first and third classes have been outlined above. Infections with the second type of virus would usually leave the nervous system unscathed, yet under suitable conditions might produce an encephalopathy from interference with the vascular system of the brain. Perhaps in hog cholera encephalomyelitis this factor is far from negligible. It is to be noted that a more or less similar classification is implicit in Seifried's description of the various encephalomyelitides (1931).

SUMMARY

After intramuscular, intradermal and subcutaneous inoculation, the pseudorabies virus reaches the central nervous system by way of the peripheral nerves, although it is circulating in the blood. Centrifugal spread from the infected nervous tissues by the neural route also occurs. After intracerebral inoculation the virus passes in the reverse direction, down the nervous axis. The Aujeszky strain invades the blood stream more readily than does the Iowa strain; but possibly with repeated passage the latter is approximating in this respect more closely the classical Aujeszky strain. After intravenous inoculation, effective with even small doses, virus is rapidly removed from the blood, and multiple infective foci are established in various organs; thence ascent of the virus by the peripheral nerves leads to infection of the central nervous system, the symptomatology differing according to whether the spinal cord or the medulla is first reached. The lack of evidence that the virus can penetrate directly the hemato-encephalic barrier deserves emphasis. When subcutaneous inoculation is practised in an area deprived of its nerve supply, the ability of the virus to invade

the blood stream permits it to establish infective foci in the various viscera, and, after a predictable delay, the course of infection resembles that following intravenous injection. The pseudorabies virus is pantropic; *i.e.*, it readily attacks cells derived from any embryonic layer. Lesions in the adrenal gland following intravenous inoculation are very like those due to herpes virus similarly introduced, this being one point of similarity in the pathogenic action of the two organisms. The relation of the pseudorabies virus to other viruses affecting the central nervous system is discussed.

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SEROLOGICAL STUDIES ON AZOPROTEINS

ANTIGENS CONTAINING AZOCOMPONENTS WITH ALIPHATIC SIDE CHAINS

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The first studies in this series included only simple aromatic amino compounds. An extension of the investigation to aliphatic substances, such as tartaric acid (1), peptides (2) and carbohydrates (3, 4), was made possible by the expedient of combining them to aromatic amines to form diazotizable compounds which could be linked to protein. Since simple aliphatic compounds have not been examined systematically with regard to their serological properties, experiments with such substances have been undertaken. The results will be presented here.

The combining of aliphatic acids with protein was effected either by the use of aminophenyl derivatives (NH_2⟨ ⟩$(CH_2)_nCOOH$) or by converting dibasic acids into aminoanilic acids (NH_2⟨ ⟩$NHCO$-$(CH_2)_nCOOH$). In addition, azoproteins containing components of the type ⟨ ⟩$(CH_2)_nCH_3$ and ⟨ ⟩$NHCO(CH_2)_nCH_3$, were prepared and used for immunization.

Materials and Methods

I. Acids: NH_2⟨ ⟩$(CH_2)_nCOOH$.—γ (*p*-aminophenyl)-butyric acid and ϵ (*p*-aminophenyl)-caproic acid were made by reduction of the corresponding nitro compounds (5). The *p*-aminophenylacetic acid used was a commercial preparation.

II. Acids: NH_2⟨ ⟩$NHCO(CH_2)_nCOOH$.—The preparation of these substances has been described in a previous paper (6). In addition the following two substances were prepared:

d,l-p-Nitromalanilic acid: This was obtained by the method used previously for optically active p-nitromalanilic acids (7). It was purified by recrystallizations from water and 25 per cent alcohol. Platelets, m.p. 178–179°C. Titration: 0.127 gm. dissolved in 80 per cent alcohol required for neutralization 5 cc. N/10 NaOH; formula $C_{10}H_{10}O_6N_2$ requires 5 cc.

d,l-p-Nitrochlorosuccinanilic acid: 5 gm. of d,l-chlorosuccinylchloride (8) (b.p. 80–81°C. at 11 mm.) dissolved in 50 cc. benzene was added to 250 cc. of a solution of 3.65 gm. of p-nitraniline in benzene at 60° and after heating on the steam bath for $2\frac{1}{2}$ hours, the mixture was kept in a closed flask for 2 days at room temperature. The precipitate was filtered off, suspended in 15 cc. of water and N NaOH added in drops until a test on litmus paper showed that no more NaOH was taken up. Upon acidification of the filtered solution with hydrochloric acid an oil separated which soon crystallized. The substance was purified by recrystallization from water and 25 per cent alcohol. Needles, m.p. 145–146°C. Analysis: $C_{10}H_9O_5N_2Cl$, calculated Cl 13.01 per cent; found 13.65 per cent. In order to determine whether the Cl occupies the same positon in the molecule as the OH group in the p-nitromalanilic acid described above, 0.273 gm. of the substance was dissolved in 10 cc. of water by the addition of 1 cc. of N NaOH, and the replacement of Cl by OH was brought about by adding 1 cc. of N alkali in portions of 0.05 cc. at 60°C., each addition being made after the alkali has been used up. After cooling and filtration from a small amount of insoluble material, the solution was acidified with hydrochloric acid. The substance crystallized in platelets like the p-nitromalanilic acid, m.p. 173–174°C.; after recrystallization from 25 per cent alcohol, m.p. 178–179°C. When mixed with an equal amount of p-nitromalanilic acid the melting point remained unchanged. A qualitative test showed no chlorine to be present. Analysis: $C_{10}H_{10}O_6N_2$, calculated N 11.02 per cent; found 10.8 per cent. It may be concluded, therefore, that in the p-nitrochlorosuccinanilic acid and p-nitromalanilic acids described the Cl and OH respectively occupy the same position relative to the carboxyl group.

III. Homologues of Aniline: NH_2⟨　⟩$(CH_2)_nCH_3$.—These compounds were made following the methods of Benz (9) for the preparation of p-aminoethylbenzene and Beran (10) for p-amino-n-octylbenzene. Ethyl, n-butyl, n-hexyl and n-octyl alcohol were heated with molar quantities of aniline and zinc chloride in sealed tubes for 8 hours at 280°. The contents of the tubes were dissolved in dilute hydrochloric acid, the solution neutralized with ammonia and an excess of ammonia added to dissolve the precipitated zinc hydroxide. The bases were extracted from the solutions with ether and fractionated by distillation at atmospheric pressure. Crude p-amino-n-butylbenzene distilled over between 230 and 260°, crude p-amino-n-hexylbenzene between 270 and 310°. They were converted into sulfates and recrystallized from 95 per cent alcohol and washed several times with alcohol until the wash liquids were colorless. After decomposition of the sulfates with sodium hydroxide, the free amino compounds were extracted with ether and distilled.

p-Amino-n-butylbenzene: B.p. 248–250°.   Analysis: calculated for $C_{10}H_{15}N$: N 9.39 per cent; found 9.33 per cent.

p-Amino-n-hexylbenzene: B.p. 279–285°.   Analysis: calculated for $C_{12}H_{19}N$: N 7.91 per cent; found 7.79 per cent.

Both substances were oils which did not crystallize upon standing in the ice box. The position of the substituents was not determined, but it can be assumed from the analogy with p-aminoethylbenzene and p-aminooctylbenzene that the substitution occurred in para-position.

IV. Homologues of p-Aminoacetanilide: $NH_2 \diagdown\diagup NHCO(CH_2)_nCH_3.$—

p-Nitrobutyr-, p-nitrocapro and p-nitrocapryl-anilide were obtained as follows: 13.8 gm. of p-nitraniline (0.1 mol) were dissolved in 80 cc. of acetone, the solution cooled and a total of 0.102 mol of the acid chloride (butyl chloride, etc.) and 0.11 mol of sodium hydroxide (25 per cent solution) was added alternately in 5 portions, shaking vigorously after each addition.   After dilution of the liquid with 4 volumes of water, enough sodium carbonate solution was added to make the solution distinctly alkaline to litmus.   The oil which separated soon crystallized upon rubbing.

p-Nitrobutyranilide: The crude product was recrystallized twice from 2 volumes of 95 per cent alcohol.   Light yellow crystals resembling octahedra, m.p. 138–139°. Analysis: calculated for $C_{10}H_{12}O_3N_2$: N 13.46 per cent; found 13.54 per cent.

p-Nitrocaproanilide: The crude product was recrystallized three times from 3 volumes of 75 per cent alcohol.   Pale yellow, long needles, m.p. 77–78°.   Analysis: calculated for $C_{12}H_{16}O_3N_2$: N 11.86 per cent; found 12.07 per cent.

p-Nitrocaprylanilide: The substance was recrystallized twice from 3 volumes of 75 per cent alcohol.   Pale yellow rhombic platelets, m.p. 82–83°.   Analysis: calculated for $C_{14}H_{20}O_3N_2$: N 10.61 per cent; found 10.88 per cent.

The amino anilides were prepared as follows: 10 gm. of finely ground nitro compound and 25 gm. of zinc dust were suspended in 250 cc. of water and a total of 350 cc. normal hydrochloric acid was added in portions of 50 cc. over a period of 1 hour with constant vigorous stirring.   (50 per cent alcohol and a normal solution of hydrochloric acid in 50 per cent alcohol were used in the reduction of the nitrocaprylanilide.)   After the nitro compound had disappeared, the solution was filtered and sodium acetate added.   The precipitate formed was redissolved in water by addition of an excess of ammonia and the solution was extracted with ether. The amino compound obtained by evaporation of the ether was recrystallized from benzene.

p-Aminobutyranilide: Yield 3.5 gm. from 10 gm. of the nitro compound.   It was recrystallized from 20 volumes of benzene.   The light colored oil, which separated on cooling, slowly crystallized in white microscopic needles, m.p. 64–65°. Analysis, after drying at 50° *in vacuo* over H_2SO_4: calculated for $C_{10}H_{14}ON_2$: N 15.73 per cent; found 15.55 per cent.

p-Aminocaproanilide: Yield 6 gm. from 10 gm. of nitro compound.   The substance was recrystallized from 5 volumes of benzene.   White microscopic needles,

m.p. 86–87°. Analysis, after drying at 60° *in vacuo* over H_2SO_4: calculated for $C_{12}H_{18}ON_2$: N 13.59 per cent; found 13.34 per cent.

p-Aminocaprylanilide: Yield 8 gm. The substance was recrystallized from 5 volumes of benzene. White microscopic needles, m.p. 99–100°. Analysis, after drying at 60° *in vacuo* over H_2SO_4: calculated for $C_{14}H_{22}ON_2$: N 11.96 per cent; found 11.73 per cent.

Antigens for Immunization.—3 millimols of the substances of the types I and II were diazotized in the usual way (2) and each diazo solution (volume 80 cc.) was added to a cold mixture of 80 cc. of a 2 per cent solution of globulin, prepared from horse serum, and 40 cc. of N sodium carbonate solution. After coupling for 1/2 hour at 0–5°, the azoproteins were precipitated by careful addition of dilute hydrochloric acid, centrifuged, washed several times with saline, dissolved in saline and brought to neutral litmus reaction by addition of normal sodium hydroxide. The final solutions contained 0.5 per cent azoprotein and 0.25 per cent phenol, added as a preservative. In the same way the substances III and IV were diazotized and coupled to horse serum, using 1.27 millimols for each 10 cc. of serum. The azoproteins were then washed with a large volume of 80 per cent alcohol and subsequently with saline. They were ground into a fine suspension and made up with saline containing 0.25 per cent phenol to a volume twice that of the serum used.

Immunization.—Batches of five rabbits each were taken for the immunization with the various antigens. Of the antigens of series I and II each rabbit received daily intravenous injections of 10 mg. of azoproteins for 6 days. One or two more courses were given at intervals of 1 week; of the antigens of series III and IV each rabbit was injected intraperitoneally with 10 to 15 cc. of the azoprotein suspension at weekly intervals. The sera were tested 7 days after the last injection.

Antigens for the Tests.—On account of the insolubility of the antigens of the types III and IV when made from serum protein, the test antigens were prepared from a solution of casein. The coupling was carried out as described above. The dilutions given in the tables are in terms of 5 per cent stock solutions, made by dissolving the azoproteins in saline with the aid of dilute sodium hydroxide and adjusting to neutral reaction. The casein-azocompounds prepared from amino-caprylanilide, aminobutylbenzene, aminohexylbenzene and aminooctylbenzene did not dissolve well, and in order to obtain a clear solution for the precipitin tests, it was necessary to add 0.2 cc. of alcohol and 0.05 cc. of N/10 sodium hydroxide to 0.1 cc. of the emulsion; when after a few minutes a clear solution was obtained, enough saline was added to give the required dilution in terms of a 5 per cent solution.

Tests.—The precipitin and inhibition tests were carried out and the strength of the reactions recorded as in former papers (1, 2).

EXPERIMENTAL

Precipitin Reactions.—The specificity of the reactions of the "anilic acid immune sera" has been dealt with in a previous communication,

TABLE I   Readings after 2 hours at room temperature and after standing overnight in the ice box.

4 drops of immune serum were added to 0.2 cc. of the diluted antigens.

Immune sera for azoproteins made from	Readings after	Azoproteins made from casein and:											
		p-Aminobenzoic acid $A^*\text{-}CO_2H$			p-Aminophenylacetic acid $A\text{-}CH_2CO_2H$			p-Aminophenylbutyric acid $A\text{-}(CH_2)_3CO_2H$			p-Aminophenylcaproic acid $A\text{-}(CH_2)_5CO_2H$		
		1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
p-Aminophenyl-acetic acid	2 hrs.	0	0	0	+ / +++	++ / +++±	+ / ++	0	0	0	0	0	0
	Night in ice box	0	0	0				f. tr.	0	f. tr.	0	0	0
p-Aminophenyl-butyric acid	2 hrs.	0	0	0	tr. / +	+ / ++	tr. / +	++± / +++±	++± / +++±	+ / +++	± / ++	+ / ++±	± / ++
	Night in ice box	0	0	0									
p-Aminophenyl-caproic acid	2 hrs.	f. tr.	0	0	± / ++±	++ / +	tr. / ±	+ / +++±	++ / +++±	± / ++±	+++ / +++±	++ / +++±	± / +++±
	Night in ice box	f. tr.	f. tr.	0									

* $A\text{-}$ represents $NH_2C_6H_4$.

2 drops of immune serum were added to 0.2 cc. of the diluted antigens.   Readings after 1 ho

Immune sera for azoproteins made from	Readings taken after	p-Aminooxanilic acid B^*-CO$_2$H			p-Aminomalonanilic acid B-CH$_2$CO$_2$H			p-Aminosuccinanilic acid B-(CH$_2$)$_2$CO$_2$H			p-Aminoglutaranilic acid B-(CH$_2$)$_3$CO$_2$H			p-Ami B
		1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100
p-Aminooxanilic acid	1 hr.	+	++	+	0	0	0	0	0	0	0	0	0	0
	Night in ice box	++	+++	++±	0	0	0	0	0	0	0	0	0	0
p-Aminosuccin-anilic acid	1 hr.	0	0	0	0	0	0	++±	++++	++	±	+	tr.	0
	Night in ice box	0	0	0	±	±	f. tr.	+++	++++	+++	±	+	±	f. tr.
p-Aminoadipan-ilic acid	1 hr.	0	0	0	0	0	0	tr.	tr.	tr.	±	+	±	++
	Night in ice box	0	0	0	0	0	0	+	+±	±	++	++	±	++++
p-Aminosuber-anilic acid	1 hr.	0	0	0	0	0	0	tr.	±	f. tr.	+	+±	+	++
	Night in ice box	0	0	0	0	0	0	±	+	tr.	++	++	+	++++

* B– represents NH$_2$C$_6$H$_4$NHCO.

† A– represents NH$_2$C$_6$H$_4$.

perature and after standing overnight in the ice box.

teins made from casein and:

p-Aminopimelanilic acid B-(CH₂)₅CO₂H			p-Aminosuberanilic acid B-(CH₂)₆CO₂H			p-Amino-benzoic acid A↑-CO₂H			p-Aminophenyl-acetic acid A-CH₂CO₂H			p-Aminophenyl-butyric acid A-(CH₂)₃CO₂H			p-Aminophenyl-caproic acid A-(CH₂)₅CO₂H		
1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
						0 0	0 0	0 0	0 0	0 0	0	0 0	0 0	0	0 0	0 0	0
0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0	0 0	0 0	0	0 0	0 0	0
0 0	0 0	0 0	0 0	0 0	0	0 0	0 0	0	f. tr. tr.	tr. ±	f. tr. tr.	tr. ±	f. tr. tr.	tr.	tr. tr.	f. tr. tr.	f. tr. tr.
+ +±	++± +++	+ +±	+ ++±	+ ++	++	tr: +±	± +±	f. tr. ±	tr. +±+±	± ++	tr. +	+± +++±	+± +++±	+ ++±	+± +++±	++± +++	± ++
++± ++++	+++± +++	++ +++	++++ +++±	++ +++±	++ +++												

in which it was shown that specific precipitin reactions can take place not only with azoproteins, but with dyes such as those prepared by coupling the diazotized aminoanilic acids to resorcinol, as well.

The reactions of the immune sera presented in Tables I and II[1] have this in common that the lower members of each series of acids react more specifically, the range of activity becoming wider with increasing length of the aliphatic side chains of the corresponding antigens. Thus the sera for oxanilic, succinanilic and phenylacetic acid display a high degree of specificity as compared with the immune sera for the compounds with longer side chains. However, with the latter immune sera, too, the strongest reactions always occur with the homologous antigens. On the whole the specificity is more pronounced in compounds of type II, which contain an NH-CO linkage, than in those of type I. The immune sera for adipanilic and suberanilic acid also showed marked group reactions with the antigens of the phenylacetic acid series. Moreover precipitation occurred between the immune sera of the phenylacetic acid series and the "anilic acid" antigens and these reactions were in all cases strongest with the highest numbers of the latter series. There is not sufficient evidence to give a final interpretation of these reactions, but it may be pointed out that phenomena, probably of a similar nature, were observed in inhibition reactions with higher aliphatic acids, which will be described below.

The immunization experiments with the compounds of series III were not very successful and only one serum each, of moderate activity, was obtained for p-aminoethylbenzene and p-aminobutylbenzene. It would probably be possible to improve the production of immune sera by modifying the technique, but this point was not further investigated. From the reactions of these sera and those for the compounds of series IV it appears that serological differences are brought about by variations in the length of the carbon chains (Tables III and IV). The "suberanilic acid immune sera" gave weak reactions with the aminobutyranilide and aminocaproanilide antigens which may be attributable to a certain correspondence in structure. No reactions of the other immune sera for substances II or those for substances I with the antigens of series III and IV were observed, nor

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[1] Reactions of other immune sera were in conformity with those recorded in the tables.

TABLE III

4 drops of immune serum were added to 0.2 cc. of the diluted antigens. Readings after 4 hours at room temperature and after standing overnight in the ice box.

Immune sera for azoproteins made from	Readings taken after	Azoproteins made from casein and:																	
		Aniline			p-Toluidine			p-Aminoethyl-benzene			p-Aminobutyl-benzene			p-Aminohexyl-benzene			p-Aminooctyl-benzene		
		1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
p-Amino-ethylben-zene	4 hrs.	0	0	0	0	0	0	0	+	0		tr.			0				
	Night in ice box	0	0	0	0	0	0	0	++	tr.		+			f. tr.				
p-Amino-butylben-zene	4 hrs.	0	0	0	0	0	0	0	0	0	tr.	+	tr.	f. tr.	f. tr.	0	0	0	0
	Night in ice box	0	0	0	0	0	0	0	tr.	tr.	tr.	+±	±	f. tr.	f. tr.	f. tr.	0	0	0

TABLE IV

From 2 to 4 drops of the immune sera were added to 0.2 cc. of the diluted antigens. Readings after 1 hour at room temperature and after standing overnight in the ice box

Immune sera for azoproteins made from	Readings taken after	Azoproteins made from casein and:											
		p-Aminoacetanilide			p-Aminobutyranilide			p-Aminocaproanilide			p-Aminocaprylanilide		
		1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500	1:100	1:500	1:2500
p-Aminoacetanilide 2 drops	1 hr.	+	+±	+	0	+	±	0	0	0	0	0	0
	Night in ice box	+	+++	+±	tr.	+±	+±	0	tr. —	f. tr.	0	f. tr.	0
p-Aminobutyranilide 3 drops	1 hr.	0	0	0	tr.	+±	+	f. tr.	±	0	0	0	0
	Night in ice box	0	+±	+±	tr.	+++	++	tr.	+±	+	0	f. tr.	0
p-Aminocaproanilide 4 drops	1 hr.	0	0	0	0	0	0	tr.	+	f. tr.	0	0	0
	Night in ice box	0	0	0	0	±	+	tr.	+±	+±	0	f. tr.	0

of the immune sera for compounds III and IV with any of the other types of antigens.

With regard to the group reactions in general the question may be raised whether they are not due in part to a breaking down of the aliphatic chain of the immunizing antigen in the animal with subsequent formation of antibodies to the resulting products. Of course, it is impossible to explain the reactions with antigens containing longer aliphatic chains than the homologous antigen in this manner. As regards the antigens with shorter chains, if the assumption were correct, one would expect, according to the theory of the so called β-oxidation, that the group reactions occur mainly with the antigens containing aliphatic chains, which differ from those present in the immunizing antigen by an even number of carbon atoms. Such a preference, however, is not noticeable in the tests with the immune sera for the anilic acids as a perusal of Table II will show.

Inhibition Tests.—Such tests furnished a confirmation of the precipitin reactions and in addition yielded new results of some consequence. The reactions presented in Table V, which were carried out with aminoanilic acids, demonstrate that in the inhibition tests, too, the strongest reactions take place with the homologous substances and that the specificity of the immune sera is less in those corresponding to the antigens with the longest aliphatic side chains. The results with the nitranilic acids were similar. When comparing the reactions of the sera for adipanilic and suberanilic acid, the greater strength of the latter immune serum must be taken into account. To obtain inhibition reactions with the dibasic acids themselves (Table VI) higher concentrations were needed than those used in the tests with the anilic acids. In this way succinic acid gave a marked inhibition reaction with the "succinanilic acid immune serum" and this reaction showed distinct specificity, since succinic acid could easily be differentiated from malonic, adipic and, though less strikingly, from glutaric acid.

However, the higher dibasic acids also showed definite inhibitions with the "succinanilic acid immune serum," increasing with the length of the carbon chain so that in the scale of reactions as presented in the table there are two maxima of inhibition, one with succinic and the other with sebacic acid. Similar effects were noticed

TABLE V

In the tests presented in Table V 0.2 cc. of the test antigen (diluted 1:500 with saline) was mixed with 0.05 cc. of a neutral solution containing 1 millimol of the substances in 10 cc.   To this 2 capillary drops of immune serum were added.   The control tube contained 0.05 cc. saline, 0.2 cc. test antigen (1:500) and immune serum.

Immune sera (2 drops) for azoproteins made from	Readings taken after	Substances used for the inhibition tests											Control
		p-Amino-oxanilic acid	p-Amino-malonanilic acid	p-Amino-succinanilic acid	p-Amino-glutaranilic acid	p-Amino-adipanilic acid	p-Amino-pimelanilic acid	p-Amino-suberanilic acid	p-Amino-benzoic acid	p-Amino-phenylacetic acid	p-Amino-phenylbutyric acid	p-Amino-phenylcaproic acid	
p-Aminooxanilic acid	1 hr. at room temperature	0	+	+	+	+	+	+	+	+	+	+	+±
p-Aminosuccinanilic acid	1 hr. at room temperature	+++	+++±	0	+++±	++++	+++	+++	++++±	+++	++++	++++	++++
p-Aminoadipanilic acid	1 hr. at room temperature	++±	++±	++	tr.	0	f. tr.	f. tr.	++±	++	++	tr.	++
p-Aminosuberanilic acid	1 hr. at room temperature	++++±	+++++±	+++	+++±	+	++	0	+++	++	+	++	++++

TABLE VI

In the tests presented in Table VI 0.2 cc. of the test antigen (diluted 1:500 with 0.15 per cent salt solution) was mixed with 4 millimols of the substances in 10 cc. To this 1 to 2 capillary drops of immune serum were added. The control tube contained 0.1 cc. of a neutral solution containing 0.1 cc. 2.4 per cent salt solution, 0.2 cc. test antigen (1:500) and immune serum. The readings were taken. (Oxalic acid was not used because of the formation of precipitate of calcium oxalate upon addition of the substances in 10 cc.) Readings between parentheses are of tests made with 0.05 cc. of a neutral solution containing 1 millimol of the substances in 10 cc.

Immune sera for azoproteins made from	Readings taken after	Substances used for the inhibition tests							
		Malonic acid	Succinic acid	Glutaric acid	Adipic acid	Pimelic acid	Suberic acid	Sebacic acid	Control
p-Aminooxanilic acid (2 drops)	15 min.	+	+	+±	+	0	0	0	+±
	1 hr.	+	+±	+±	+	0	0	0	++
	Night in ice box	++	+±	++	+±	±	±	tr.	+++
p-Aminosuccinanilic acid (1 drop)	15 min.	tr.	0	0	+	f. tr.	tr.	0	++
	1 hr.	+	0	±	+±	+±	+±	0	++±
	Night in ice box	++	tr.	+	++±	+++±	+±	+±	++++±
p-Aminoadi-panilic acid (2 drops)	15 min.	+±	+	+	0	0 (±)	0 (tr.)	0 (0)	+++±
	1 hr.	+++±	++	++±	f. tr.	0 (±)	0 (tr.)	0 (f. tr.)	+++±
	Night in ice box	+++++±	++++±	++±	±	0	0	0	++++++±
p-Aminosuber-anilic acid (1 drop)	15 min.	+±	+	+	0	0 (+)	0	0	++ (0)
	1 hr.	+++±	++	++±	0	0 (+±)	0	0	+++ (tr.)
	Night in ice box	+++++±	++++±	+++±	±	0	0	0	++++++

with the immune sera for phenylacetic acid and its homologues; c.g., the precipitation of the immune sera for phenylacetic acid was inhibited considerably by caprylic, caproic, sebacic and suberic acid (cf. 11). The inhibitions by acids with longer chains are non-specific in so far as acids which are structurally further removed from the homologous antigen react more intensely with the immune sera than closely related ones. Evidently, these inhibition phenomena are dependent on certain physicochemical properties common to salts of acids with long aliphatic chains. Nevertheless, the reactions have some degree of specificity. It is true that an inhibitory action of the sodium salts of sebacic and also of suberic acid on the precipitation by various immune sera, such as precipitins for horse or pig serum, can be demonstrated by using sufficiently concentrated solutions of these salts, but these effects are distinctly less pronounced than the inhibitions with the anilic acid immune sera. Consequently, the inhibitions depend to a certain extent on the similarity of the chemical composition of the inhibiting substances and the antigens, and the reactions constitute, as it were, a transition between specific and non-specific phenomena.

The fact that succinic acid by itself shows specific reactions with antibodies is interesting because of the simple composition of the compound and should be emphasized in connection with a criticism by Berger (12) concerning the applicability of the azoprotein method for studying the serological specificity of simple compounds. As will be shown presently, succinic acid can be serologically distinguished not only from the homologous dibasic acids but also from malic, tartaric and asparaginic acid. Similarly in former experiments, using sera for azoproteins prepared with the stereoisomeric *p*-aminotartranilic acids it was possible to differentiate the three stereoisomeric tartaric acids from one another and from succinic acid (1). Some of the differences observed were only small, but since they were definite this does not nullify the results. Moreover, the striking specificity of the inhibition reactions with the stereoisomeric nitro- and aminotartranilic acids can depend only upon the tartaric acid part of the molecule since the other moiety is the same in the various compounds. The same reasoning holds true for the observations on acylated peptides (2) and for the inhibition reactions with aminophenol-glucosides and -galactosides carried out by Avery and Goebel (3, 4).

TABLE VII

Technique as described for Table V

Immune sera (1 drop) for azoprotein made from p-Aminosuccinanilic acid — Readings taken after:

Substances used for inhibition tests	1 hr.	Night in ice box
p-Nitrosuccinanilic acid	0	0
d,l,-p-Nitrochlorosuccinanilic acid	f. tr.	+
d,l,-p-Nitromalanilic acid	++	++++
d,l,-p-Nitrotartranilic acid	++	++++
Control	++±	++++

Immune sera (4 drops) for azoprotein made from p-Aminophenylacetic acid — Readings taken after:

Substances used for inhibition tests	1 hr.	Night in ice box
Phenylacetic acid	0	tr.
d,l-Mandelic acid	±+	++
Control	++	++±

Immune sera (4 drops) for azoprotein made from p-Aminophenylbutyric acid — Readings taken after:

Substances used for inhibition tests	15 min.	1 hr.	Night in ice box
Phenylbutyric acid	0	tr.	+
Phenyloxybutyric acid	±+	++	+++±
Phenylisocrotonic acid	f. tr.	±±	++
Control	++	+++	+++

Tests with Substituted Aliphatic Acids.—A few substances were examined in order to investigate the influence of various substituents on the reactions. From Table VII it is seen that the substitution of hydrogen atoms in the aliphatic chains causes a serological change which in the tests with the anilic acids is greater in the OH than the Cl substituted compounds, and still more pronounced when two hydro-

TABLE VIII

Technique as described for Table VI

Immune serum for azoprotein made from	Readings taken after	Substances used for the inhibition tests										
		Succinic acid	l-Malic acid	d-Tartaric acid	Asparaginic acid	Itaconic acid	Succinic acid mono-methyl ester	Succinic acid mono-amide	Succinimide	Butyric acid	Crotonic acid	Control
p-Aminosuc-cinanilic acid (1 drop)	15 min.	0	+	+±	++	0	0	0	++	+	+	++
	1 hour	0	+±	++	++	0	tr.	0	+++	+±	+±	+++±
	Night in ice box	tr.	++++	++++	++++	0	+±	0	++++	+++	++++±	++++

TABLE IX

Technique as described for Table VI

Immune serum for azoprotein made from	Readings taken after	Substances used for the inhibition tests						
		Succinic acid	Fumaric acid	Maleic acid	Mesaconic acid	Citra-conic acid	Acetylene dicarboxy-lic acid	Control
p-Amino-succin-anilic acid (1 drop)	15 min.	0	+±	0	+±	0	+±	++
	1 hr.	0	++	0	++	0	++	++±
	Night in ice box	tr.	++++	0	++++	0	++++±	++++

gens were replaced by OH groups. Table VIII shows the marked difference between succinic acid on the one hand and malic, tartaric and asparaginic acid on the other. Succinimide which contains no free carboxyl group caused no inhibition, in contrast to the monomethyl ester and the monoamide.

A more extensive study on the influence of substituents seems desirable in order to establish their relative serological significance. Such

an investigation would also be of interest with regard to the views recently put forth by Erlenmeyer and Berger (13) on the identical serological behavior of "isosteric" atoms or radicals.

Serological Differentiation of cis-trans Isomers.—In order to complete a preliminary note (7) on this subject inhibition reactions of a serum for "succinic acid" azoproteins are presented in Table IX. Strong inhibition effects were produced by succinic, maleic and citraconic acid, the two latter being cis compounds whereas the corresponding trans compounds namely fumaric and mesaconic acid had no inhibitory action. It follows that the cis-trans isomerism is serologically significant just as is the isomerism which depends on asymmetric carbon atoms.

As an explanation of the behavior of maleic acid in contrast to fumaric acid one could assume that succinic acid, to which the antibodies correspond, can exist in a spatial configuration similar to that of the cis compound (14), or that the antibody may adjust itself to the cis structure.

SUMMARY

A study was made of the specificity of artificial compound-antigens containing aliphatic chains.

Striking specificity was exhibited in the reactions of compounds with short chains containing a carboxyl group; for instance succinic acid could be differentiated from malonic or glutaric acid which contain one fewer and one more carbon atom respectively. With the substances containing longer chains, reactions were observed which, although specific to a certain extent, appear to depend mainly on the general physicochemical properties of long aliphatic chains.

With a limited number of substances the influence of substituents —halogen, OH, NH_2—on the serological specificity was investigated.

By means of inhibition reactions it was possible to demonstrate the serological specificity of cis-trans isomers such as maleic and fumaric acid.

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ON THE SEROLOGICAL SPECIFICITY OF PEPTIDES.   II

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The results of serological tests with azoproteins prepared from peptides of known structure, namely glycyl-glycine, glycyl-leucine, leucyl-glycine and leucyl-leucine, were reported in a previous communication (1).   By means of precipitin and inhibition tests it was found that these peptides were serologically distinct and that their specificity was determined primarily by the terminal amino acid and to a lesser extent by the second amino acid.   These experiments were taken up again and extended to other peptides.

Preparation of Peptides and Their Nitrobenzoyl and Aminobenzoyl Derivatives

The following peptides were prepared according to the methods given in the papers referred to: diglycyl-glycine, triglycyl-glycine, tetraglycyl-glycine, di-*d,l*-leucyl-glycyl-glycine (2); diglycyl-*d,l*-leucine, triglycyl-*d,l*-leucine (3); glycyl-*d,l*-leucyl-glycine (4); *d,l*-leucyl-glycyl-glycine (5); *d,l*-leucyl-triglycyl-glycine (6).

Chloracetyl-d,l-Leucyl-Glycyl-Glycine.—30 gm. of *d,l*-leucyl-glycyl-glycine were dissolved in 124 cc. of N sodium hydroxide and the solution was cooled to −5°C. 28 gm. of chloracetylchloride and 76 cc. of 5 N sodium hydroxide were added alternately in 5 equal portions with good cooling and vigorous shaking.   The solution was made strongly acid to Congo red by addition of 5 N hydrochloric acid and extracted several times with ethyl acetate containing 10 per cent of alcohol.   The ethyl acetate solution was dried with anhydrous sodium sulfate and evaporated. The remaining oil crystallized upon stirring with ether.   Yield 31 gm.   It was recrystallized by dissolving in 20 parts of ethyl acetate and allowing the solution to stand in the ice box for 1 day.   Yield 20 gm.   Microcrystalline, m.p. 149–150°.[1] Analysis: After drying at 100° *in vacuo* over CaCl₂; calculated for $C_{12}H_{20}O_5N_3Cl$: N 13.07, found 13.12; Cl 11.02, found 10.90.

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[1] The melting points were made with the analyzed substances but were not checked by further purification.

Glycyl-d,l-Leucyl-Glycyl-Glycine.—A solution of 1 part of chloracetyl-*d,l*-leucyl-glycyl-glycine in 10 parts of ammonium hydroxide (28 per cent) was heated in a sealed tube at 100°C. for $1\frac{1}{2}$ hours. The solution was evaporated to dryness *in vacuo*, the residue dissolved in water, treated with a solution of silver sulfate to remove Cl⁻, and subsequently with hydrogen sulfide. After removal of silver sulfide and evaporation *in vacuo* to a small volume just enough barium hydroxide was added to free the solution from sulfates. The solution was evaporated to dryness *in vacuo*, the material dissolved in a very small amount of water, 10 volumes of absolute alcohol and 20 volumes of ether were added and the sticky precipitate was rubbed with a mixture of 1 part of absolute alcohol and 2 parts of dry ether until it became granular. Yield 2.4 gm. from 3 gm. of the chloracetyl compound. Amorphous, decomposes when heated above 120°. Analysis: After drying at 100° *in vacuo* over CaCl₂; calculated for $C_{12}H_{22}O_5N_4$: N 18.51, found 18.40.

Chloracetyl-Glycyl-d,l-Leucyl-Glycyl-Glycine.—This substance was prepared from glycyl-*d,l*-leucyl-glycyl-glycine as described for chloracetyl-*d,l*-leucyl-glycyl-glycine. The ethyl acetate solution was evaporated *in vacuo* and absolute alcohol was added. A white insoluble residue was separated from the colored alcoholic solution and recrystallized by dissolving in 40 parts of 95 per cent alcohol and allowing the solution to stand in the ice box overnight. Yield 6 gm. from 12 gm. of peptide. Small platelets, m.p. 195–196°C. Analysis: After drying at 100° *in vacuo* over CaCl₂; calculated for $C_{14}H_{23}O_6N_4Cl$: N 14.80, found 14.59; Cl 9.37, found 9.27.

Diglycyl-d,l-Leucyl-Glycyl-Glycine.—1 part of chloracetyl-glycyl-*d,l*-leucyl-glycyl-glycine was heated in a sealed tube with 10 parts of ammonium hydroxide (28 per cent) for $1\frac{1}{2}$ hours at 100°C. The solution was evaporated *in vacuo*, the peptide redissolved in 2 parts of water and 20 volumes of absolute alcohol were added. After standing in the ice box the precipitated peptide was centrifuged off, repurified in the same manner and washed with alcohol. Yield 3.5 gm. from 6 gm. chloracetyl compound. Not distinctly crystalline, decomposes above 215°. Analysis: After drying at 100°C. *in vacuo* over CaCl₂, calculated for $C_{14}H_{25}O_6N_5$: N 19.49, found 19.15.

α-Bromisocaproyl-Dileucyl-Glycyl-Glycine (Inactive).—This substance was prepared from dileucyl-glycyl-glycine (inactive) and *d,l*-α-bromisocaproylchloride according to the method described by Fischer for the preparation of α-bromisocaproyl-leucyl-glycyl-glycine (2). After completion of the acetylation the solution was acidified with hydrochloric acid, and the precipitate washed with petrol ether. It was recrystallized from 25 parts of 40 per cent alcohol. Yield 10 gm. from 10 gm. of peptide. Microscopic needles, m.p. 192–193° Analysis: After drying at 100°C. *in vacuo* over CaCl₂; calculated for $C_{22}H_{39}O_6N_4Br$: N 10.50, found 10.62.

Trileucyl-Glycyl-Glycine (Inactive).—10 gm. of α-bromisocaproyl-dileucyl-glycyl-glycine (inactive) was heated with 50 cc. of ammonium hydroxide (28 per cent) in a sealed tube at 100° for $1\frac{1}{2}$ hours. After evaporation on the steam bath the sub-

stance was stirred with absolute alcohol, the alcohol evaporated and the peptide suspended in 100 cc. absolute alcohol and kept in the ice box overnight. It was extracted a second time with absolute alcohol. Yield 5.7 gm. Amorphous, turns dark above 210°. Analysis: After drying at 100°C. *in vacuo* over $CaCl_2$; calculated for $C_{22}H_{41}O_6N_5$: N 14.87, found 14.60.

Chloracetyl-Triglycyl-d,l-Leucine.—This substance was prepared from trigylcyl-d,l-leucine and chloracetyl chloride in the usual way (3). The chloracetyl peptide separated from the reaction mixture upon acidification and was recrystallized from 5 parts of water. Yield 18 gm. from 30 gm. of peptide. Amorphous, m.p. 185–187°. Analysis: After drying at 100° *in vacuo* over $CaCl_2$; calculated for $C_{14}H_{23}O_6N_4Cl$: N 14.80, found 14.74.

Tetraglycyl-d,l-Leucine.—A solution of 10 gm. of chloracetyl-triglycyl-d,l-leucine in 250 cc. of 28 per cent ammonium hydroxide was kept at 37° for 3 days. Ammonia was removed by evaporation to dryness *in vacuo* and the substance was purified by the silver sulfate method. The solution of the peptide was evaporated to dryness *in vacuo*, the substance was dissolved in 15 cc. of water, 300 cc. of absolute alcohol were added and subsequently 3 volumes of dry ether to precipitate the peptide. Yield 4 gm. from 6 gm. of chloracetyl compound. The substance proved to be impure (calculated for $C_{14}H_{25}O_6N_5$: N 19.49, found 18.27). It was converted into the nitrobenzoyl derivative which crystallized easily and gave a correct analytical value for N.

The preparation of the *p*-nitrobenzoyl and *p*-aminobenzoyl derivatives of the following amino acids and peptides has been described previously (1): glycine, d,l-leucine, glycyl-glycine, glycyl-d,l-leucine, d,l-leucyl-glycine, d,l-leucyl-d,l-leucine A.

The nitrobenzoylation of the other peptides was in general made in the following manner.

A solution of 0.1 mol of the peptide in 600 cc. of 10 per cent sodium bicarbonate was mixed with 300 cc. of chloroform and a total of 55.5 gm. of finely ground *p*-nitrobenzoyl chloride (0.3 mol) was added in 5 equal portions over a period of $1\frac{1}{2}$ hours with vigorous shaking at room temperature. Ether was added and the aqueous solution, after separation from the ether-chloroform mixture, was filtered and made acid to Congo red by addition of hydrochloric acid. The precipitate was filtered off, washed with water and dried at 45° *in vacuo*. (The precipitate of the crude nitrobenzoyl derivative separated either immediately or after a longer time, up to 1 or 2 days, at low temperature). The substances were finely ground and extracted several times with boiling ether to remove *p*-nitrobenzoic acid. They were then dissolved in water by addition of alkali, reprecipitated with acid and after drying again extracted with ether.

The *p*-aminobenzoyl peptides were obtained by reduction of the *p*-nitrobenzoyl peptides as follows:

The nitrobenzoyl peptides, dissolved in about 3 parts of water by addition of a slight excess of ammonium hydroxide and heating, if necessary, were added to a

hot solution of ferrous sulfate, 7 aq. (6.5 mols for each mol of nitrobenzoyl peptide) in 2.5 parts of water. A 28 per cent ammonia solution (10 cc. for each 12 gm. of ferrous sulfate, 7 aq.) was added in 5 equal portions over a period of 10 minutes, shaking well with each addition. After heating on the steam bath for 15 minutes the ferric hydroxide was removed by filtration and the solution evaporated *in vacuo* to a small volume. The subsequent procedure varied somewhat for the different substances.

p-Nitrobenzoyl-Diglycyl-Glycine.—Recrystallized by dissolving in 170 parts of boiling 70 per cent alcohol and allowing the solution to stand in the ice box overnight. Yield 9 gm. from 6 gm. of peptide. Large needles, turns dark above 245°. Analysis:[2] Calculated for $C_{13}H_{14}O_7N_4$: N 16.57, found 16.45.

p-Aminobenzoyl-Diglycyl-Glycine.—The concentrated solution was made weakly acid to Congo red with hydrochloric acid and kept in the ice box overnight. The precipitate was recrystallized from 25 parts of water. Yield 3.2 gm. from 4 gm. of nitro compound. Rosettes of small needles, turns dark above 245°. Analysis: Calculated for $C_{13}H_{16}O_5N_4$: N 18.19, found 17.91.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine.—Recrystallized from 40 parts of 30 per cent alcohol. Yield 6 gm. from 6 gm. of the peptide. Microscopic platelets, m.p. 175–176°. Analysis: Calculated for $C_{17}H_{22}O_7N_4$: N 14.21, found 14.11.

p-Aminobenzoyl-Diglycyl-d,l-Leucine.—The concentrated solution was made weakly acid to Congo red and kept in the ice box overnight. The precipitate was recrystallized from 25 parts of water. Yield 4 gm. from 6 gm. of nitrobenzoyl compound. Microscopic needles, m.p. 176–177°. Analysis: Calculated for $C_{17}H_{24}O_5N_4$: N 15.38, found 15.34.

p-Nitrobenzoyl-Glycyl-d,l-Leucyl-Glycine.—Recrystallized from 20 parts of 30 per cent alcohol. Yield 9 gm. from 10 gm. of peptide. Rosettes of microscopic needles, m.p. 204–205°. Analysis: Calculated for $C_{17}H_{22}O_7N_4$: N 14.21, found 14.13.

p-Aminobenzoyl-Glycyl-d,l-Leucyl-Glycine.—To the concentrated solution 10 volumes of alcohol were added, ammonium sulfate was filtered off, the filtrate evaporated to dryness *in vacuo*, the residue dissolved in a small amount of water and after addition of hydrochloric acid to weak acidity to Congo red, the solution was concentrated in a vacuum desiccator until the aminobenzoyl peptide separated. It was recrystallized from 4 parts of water. Yield 3.4 gm. from 4.8 gm. of nitrobenzoyl compound. Microscopic platelets, m.p. 179–181°. Analysis: Calculated for $C_{17}H_{24}O_5N_4$: N 15.38, found 14.91.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine.—Prepared by nitrobenzoylation as described by Abderhalden, Dinerstein and Genes (7). M.p. 167–168°. Analysis: Calculated for $C_{17}H_{22}O_7N_4$: N 14.24, found 14.04.

p-Aminobenzoyl-d,l-Leucyl-Glycyl-Glycine.—After the reduction of nitrobenzoyl-

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[2] For analysis the substances were dried at 100° *in vacuo* over $CaCl_2$ unless differently stated.

d,l-leucyl-glycyl-glycine the solution was evaporated *in vacuo* to a small volume, 10 volumes of alcohol were added, ammonium sulfate filtered off and the alcoholic solution evaporated to dryness *in vacuo*. The substance was dissolved in a small amount of alcohol and precipitated by addition of ether. After drying, it was dissolved in 2 parts of water and enough 5 N hydrochloric acid was added to make the solution weakly acid to Congo red. After being kept for 2 days at room temperature, the precipitate was filtered off and recrystallized from 5 volumes of water, using norit for decolorizing. Crystallization was allowed to take place for 2 days at room temperature. Yield 4.2 gm. from 8.4 gm. of nitrobenzoyl compound. Irregular platelets, m.p. 179–180°. Analysis: Calculated for $C_{17}H_{24}O_5N_4$: N 15.38, found 15.31.

p-Nitrobenzoyl-Triglycyl-Glycine.—Recrystallized from 20 parts of 50 per cent alcohol. Yield 11 gm. from 10 gm. of peptide. Microscopic needles, decomposes at about 230°. Analysis: Calculated for $C_{15}H_{17}O_8O_5$: N 17.72, found 17.49.

p-Aminobenzoyl-Triglycyl-Glycine.—The concentrated solution was made weakly acid to Congo red. The substance was reprecipitated from an alkaline solution by addition of acid, washed with water, 50 per cent alcohol and finally with absolute alcohol and ether. Upon cooling a hot aqueous solution it separated in the form of granules. Yield 4.2 gm. from 6 gm. of nitro compound. Decomposed without melting when heated above 250°. Analysis: Calculated for $C_{15}H_{19}O_6N_5$: N 19.18, found 18.83.

p-Nitrobenzoyl-Triglycyl-d,l-Leucine.—Recrystallized from 10 parts of 30 per cent alcohol. Yield 6.2 gm. from 6 gm. of peptide. Microscopic needles, begins to sinter above 95°, decomposes at about 220°. Analysis: After drying at 80° *in vacuo* over H_2SO_4, calculated for $C_{19}H_{25}O_8N_5$: N 15.52, found 15.41.

p-Aminobenzoyl-Triglycyl-d,l-Leucine.—The concentrated solution was made weakly acid to Congo and kept at room temperature overnight. The precipitate was redissolved in 5 parts of water and crystallization was allowed to take place in the ice box. Yield 1.9 gm. from 2.7 gm. of nitrobenzoyl peptide. Microscopic needles, m.p. 176–178°. Analysis: Calculated for $C_{19}H_{27}O_6N_5$: N 16.65, found 16.28.

p-Nitrobenzoyl-Glycyl-d,l-Leucyl-Glycyl-Glycine.—Nitrobenzoylation was carried out in a freezing mixture as in the case of p-nitrobenzoyl-d,l-leucyl-glycyl-glycine. The nitrobenzoyl compound was precipitated from the cold alkaline solution by acidification with hydrochloric acid. It was freed from p-nitrobenzoic acid by repeated extractions with ether following the general procedure described above, dissolved in 40 parts of boiling water and allowed to crystallize in the ice box. Yield 6 gm. from 10 gm. of peptide. Clumps of microscopic needles, decomposes above 220°. Analysis: Calculated for $C_{19}H_{25}O_8N_5$: N 15.52, found 15.44.

p-Nitrobenzoyl-Tetraglycyl-Glycine.—Recrystallized from 200 parts of 50 per cent alcohol. Yield 9 gm. from 10 gm. of peptide. Rosettes of microscopic needles, decomposes above 240°. Analysis: Calculated for $C_{17}H_{26}O_9N_6$: N 18.59, found 18.38.

p-Aminobenzoyl-Tetraglycyl-Glycine.—Redissolved with alkali and precipitated with acid, washed with water, 50 per cent alcohol and finally with absolute alcohol and ether. Yield 5 gm. from 7 gm. of nitrobenzoyl compound. Amorphous, decomposes above 270°. Analysis: Calculated for $C_{17}H_{22}O_7N_6$: N 19.92, found 19.69.

p-Nitrobenzoyl-Tetraglycyl-Leucine.—This substance was prepared by nitrobenzoylation of impure tetraglycyl-leucine in the usual way in a solution of 10 per cent sodium bicarbonate and adding the *p*-nitrobenzoyl chloride dissolved in benzene. The alkaline solution was made weakly acid to Congo red and kept in the ice box for 1 hour. After removal of the precipitate the solution was made strongly acid to Congo red by the addition of hydrochloric acid and was kept in the ice box for 2 days. The precipitate was filtered, washed with cold water, dried and freed from *p*-nitrobenzoic acid by extractions with ether. It was recrystallized from 20 parts of water. Yield 4.2 gm. from 7.2 gm. of peptide. Microcrystalline, no definite crystal form, m.p. 191–193°. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.44.

p-Aminobenzoyl-Tetraglycyl-d, l-Leucine.—When the solution was concentrated after the reduction and made weakly acid to Congo red, the substance separated as an oil which solidified upon cooling. It was freed from ammonium sulfate by dissolving in hot alcohol and the alcoholic solution was evaporated to dryness *in vacuo*. The substance was redissolved in 2 parts of water and kept in the ice box until some brownish material separated and the colorless solution after filtration was evaporated to dryness in a vacuum desiccator. Yield 0.8 gm. from 2 gm. of nitrobenzoyl peptide. Softens at 165°. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.63.

p-Nitrobenzoyl-Diglycyl-d, l-Leucyl-Glycyl-Glycine.—Nitrobenzoylation was carried out as for the preparation of *p*-nitrobenzoyl-glycyl-*d*,*l*-leucyl-glycyl-glycine. The substance was precipitated from the alkaline solution by acidification, dried and freed from *p*-nitrobenzoic acid by extractions with ether. It was dissolved in 15 parts of boiling water and the precipitate was filtered after the solution had been kept overnight in the ice box. Yield 7 gm. from 10 gm. of peptide. Amorphous, m.p. 143–145°. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.30.

p-Aminobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine.—The concentrated solution obtained after reduction of the nitrobenzoyl peptide was freed from ammonium sulfate by addition of alcohol, and the substance after evaporation of the alcohol was redissolved in boiling absolute alcohol and precipitated from the filtered alcoholic solution by addition of 5 volumes of ether and dried. To 2 gm. dissolved in 1 cc. water 1 cc. of glacial acetic acid was added and the aminobenzoyl peptide was precipitated from the solution by the addition of acetone. Yield 1.8 gm. from 3.8 gm. of nitrobenzoyl compound. Softens at about 120°. Analysis: After drying at 60° *in vacuo* over H_2SO_4, calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.71.

p-Nitrobenzoyl-d,l-Leucyl-Triglycyl-Glycine.—Recrystallized from 40 parts of 30 per cent alcohol. Yield 9.5 gm. from 10 gm. of peptide. Rosettes of microscopic needles, decomposes above 225°. Analysis: After drying at 50° *in vacuo* over H_2SO_4, calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.31.

p-Aminobenzoyl-d,l-Leucyl-Triglycyl-Glycine.—The solution of the ammonium salt of the aminobenzoyl peptide was worked up as in the case of *p*-aminobenzoyl-diglycyl-*d*,*l*-leucyl-glycyl-glycine, and the substance was precipitated from a solution in 95 per cent alcohol by addition of ether. After drying, 2 gm. were dissolved in 1 cc. water, 1 cc. of glacial acetic acid was added and the amino compound was precipitated by the addition of acetone. It was redissolved in 10 cc. of absolute alcohol, the solution was freed from a small amount of insoluble material and the substance precipitated by addition of 70 cc. of acetone. Yield 1.3 gm. from 3 gm. of nitrobenzoyl compound. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.81.

p-Nitrobenzoyl-Trileucyl-Glycyl-Glycine (Inactive).—Nitrobenzoylation was carried out as in the case of *p*-nitrobenzoyl-glycyl-glycyl-*d*,*l*-leucyl-glycyl-glycine. The substance was precipitated from the solution by acidification and extracted with ether. Recrystallized from 40 parts of 70 per cent alcohol. Yield 8.5 gm. from 10 gm. of peptide. Rosettes of microscopic needles, m.p. 222–224°, with darkening. Analysis: Calculated for $C_{29}H_{44}O_9N_6$: N 13.55, found 13.62.

p-Aminobenzoyl-Trileucyl-Glycyl-Glycine (Inactive).—The nitrobenzoyl peptide was converted into the amino compound by dissolving 2.5 gm. in 70 cc. of 60 per cent alcohol containing 1.5 cc. concentrated ammonium hydroxide and reducing with ferrous sulfate, as described above. After heating on the steam bath the solution was filtered, the ferric hydroxide precipitate washed with hot 50 per cent alcohol and the combined solutions concentrated *in vacuo* to a small volume. The amorphous substance was filtered off after addition of sufficient hydrochloric acid to make the liquid weakly acid to Congo red. It was dissolved in a small amount of water by addition of alkali, the solution was made acid to Congo red with hydrochloric acid, some acid-insoluble material was removed by centrifugalization and the amino compound was reprecipitated from the solution with the required amount of alkali. Yield 1.2 gm. from 2.4 gm. of nitrobenzoyl compound. Amorphous. Softens at 165°. Analysis: Calculated for $C_{29}H_{46}O_7N_6$: N 14.24, found 14.20.

p-Nitrobenzoyl-Glutathione.—To a solution of 2.5 gm. of glutathione in 30 cc. of water 6 gm. of sodium bicarbonate were added and subsequently 4.3 gm. of *p*-nitrobenzoyl chloride dissolved in benzene in 5 equal portions. The mixture was shaken vigorously for 1½ hours. After filtration the aqueous solution was made acid to Congo red with hydrochloric acid, kept in the ice box overnight and the substance after drying was freed from *p*-nitrobenzoic acid by extractions with ether. Yield 2 gm. Amorphous. Softens at 140°. Analysis: After drying at 50° *in vacuo* over CaCl₂; calculated for the reduced form, $C_{17}H_{20}O_9N_4S$: S 7.02, found 7.03.

p-Aminobenzoyl-Glutathione.—After reduction of the nitro compound the solution was concentrated *in vacuo* to a small volume. Upon addition of hydrochloric acid a sticky yellow precipitate was formed which was separated from the liquid and redissolved in water by addition of sodium hydroxide. The amino compound was precipitated from the neutral solution in the form of a copper salt by addition of

copper sulfate. After filtration and washing with water the copper salt was suspended in water and decomposed with hydrogen sulfide. After filtration the copper sulfide was extracted with hot alcohol. The aqueous solution and the alcoholic extract were joined and evaporated to dryness, the substance was dissolved in alcohol and precipitated with 6 volumes of ether. Yield 1.2 gm. from 2.7 gm. of nitrobenzoyl compound. Amorphous, decomposes without melting above 105°. Analysis: After drying at 80° *in vacuo* over H_2SO_4; calculated for the reduced form, $C_{17}H_{22}O_7N_4S$: N 13.14, S 7.51; found N 12.92, S 7.58.

Serological Tests

Preparation of Antigens.—The antigens were made as described previously (1).

The azoproteins used for immunization were purified by repeated washings with saline, omitting the precipitation with alcohol. Some of the antigens, namely those used for the production of the immune sera GL No. 2 and LG No. 2 were prepared with one-half of the indicated quantity of the aminobenzoyl peptides.

Immunization and Tests.—The immunization was carried out as described (8). 2 cc. of a solution containing 10 mg. in 1 cc. were used per injection.

On repeating precipitin tests with new GL and LG immune sera,[3] these sera appeared to be less specific than those described (1), and showed stronger cross-reactions between peptides containing the same terminal amino acid; *e.g.*, the GL serum with L_2 antigen and the LG serum with G_2 antigen. One of the LG sera gave a faint group reaction with the L_2 antigen (9), and a more marked precipitation was observed with a G_2 antigen and a GL serum. These results could not be explained entirely by the greater strength of some of the sera and may be due to the individually different response of the immunized animals, or possibly to the change in technique of preparing the immune sera.

Reactions with these and other immune sera on a series of "peptide-" and "amino acid-azoproteins" are summarized in Table I. In most cases distinct group reactions occurred whenever the antigens tested contained peptides with the same terminal amino acid as that present in the homologous azoprotein, and the reactions were, as a rule, the stronger the greater the similarity in structure of the terminal part of the peptide chain. On the other hand, in a number of combina-

[3] As in the previous paper the peptides will be designated by abbreviations such as G for glycine, G_2 for glycyl-glycine, GL for glycyl-leucine, etc.

TABLE I

To 0.2 cc. of the 1:500 diluted antigens (prepared with chicken serum) were added 2 capillary drops of immune serum. The dilution was made from a 5 per cent stock solution. The readings were taken after the tests had stood for 1 hour at room temperature.

Immune sera for	Test antigens prepared from chicken serum and the aminobenzoyl derivatives of																	
	G	L	G₁	GL	LG	L₁	G₂	G₂L	GLG	LG₂	G₃	G₁L	G₄	G₄L	GLG₃	LG₄	L₃G₃	Gluta-thione
G	+++±	0	+	0	+	0	+	0	++±	+	+	0	+	0	±±	+±	0	tr.
L	0	++	0	++	0	±±	0	++	0	0	0	++	0	+±±	0	0	0	0
Gl. No. 1	0	++	f. tr.	++	0	±±	0	++	0	0	0	++	0	+±±	0	0	0	0
Gl. No. 2	+	++	+	+	0	±±	0	++±	0	0	0	+++±	0	+++	0	0	0	+
LG No. 1	+	0	+±	0	++	0	+±	0	++	+	+±	0	+±	0	++	++	tr.	++
LG No. 2	+	0	+++	0	++	0	+++	0	++	++	+++	0	+++	+++±	+±	++	±±	++±
G₂ No. 1	±	0	tr.	0	0	0	+±	0	++±	tr.	+	0	+	0	±±	+	+	f. tr.
G₂ No. 3	0	0	+++	0	0	0	+++	+	+±	+++	+±	0	+±	0	±±	+±	0	0
LG₂ No. 1	tr.	0	+++	0	±±	0	+++±	0	0	+++	+	0	+	0	+	+	+±±	0
LG₂ No. 2	f. tr.	0	+±	0	0	0	±	0	0	+++	+±	0	+±	0	+++±	+	±±	++
Glutathione	tr.	0	±±	0	f. tr.	0	±±	0	±±	tr.	tr.	0	tr.	0	f. tr.	tr.	0	±±

Tests with other dilutions of the antigens gave essentially similar results.

tions there were no or faint cross-reactions, although the substances tested have the same terminal amino acid (carrying the free carboxyl group) as the homologous antigens, *e.g.*: immune serum G + antigen L_3G_2; immune serum G_3 + antigens LG and glutathione; immune serum LG_2 + antigens G and glutathione; immune serum glutathione + antigens LG, G_2LG_2, L_3G_2.[4]   Similarly, there was a marked difference in the reactions of the two tripeptides GLG and LG_2 when tested with LG_2 immune sera, and it is worth noting that the (pentapeptide) L_3G_2 antigen gave rather weak reactions with the immune sera LG and G_3, whereas it reacted distinctly with the immune serum for LG_2 to which it is more closely related chemically.   Some overlapping reactions apparently were caused by the correspondence of amino acids not occupying the terminal position in the peptide chain; *e.g.*, one immune serum G_3 reacted moderately with the antigens G_2L, G_3L, G_4L.

In the experiments presented the only immune sera used were two sera for di- and three for tripeptides and the peptides used (apart from glutathione) were built up solely from glycine and leucine. These precipitin tests, still limited in extent, did not, on the whole, demonstrate great serological diversity.   Significantly different were the results of inhibition tests carried out with nitrobenzoyl derivatives of the peptides.   The readings are given in Table II.   The data presented in Table II reveal a striking degree of specificity.   Thus the sera GL and LG show marked group reactions only with the peptides which are very closely related in structure, namely the LG serum with the substance GLG and the GL serum with G_2L, G_3L and G_4L, but even these reactions are considerably weaker than the reactions with the homologous substances.   Likewise, with the tripeptide sera G_3 (No. 1) and LG_2 marked cross-reactions occurred only when the structure of the substances tested was identical with that of the homologous substance with regard to the three amino acids at the free end of the chain.   For instance, the sera G_3 distinguish between the substances LG_2 and G_3 or G_2LG_2 and G_5 and the serum LG_2 between GLG_2 and G_4, etc.

The greater specificity of the inhibition reactions, as compared to

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[4] The glutathione immune serum did not react on an azoprotein made with glutaminic acid.

TABLE II

For the inhibition tests 0.2 cc. of the chicken serum antigens (diluted 1:500) was mixed with 0.05 cc. of a neutral solution of the nitrobenzoyl derivatives of the peptides (concentration 0.25 millimol in 10 cc.). To this 2 capillary drops of homologous immune serum were added. The control tube contained only antigen and immune serum. Readings taken after 5 minutes and 1 hour are presented in the table on the first and second line respectively.

Immune sera for	G	L	G_3	Gl.	LG	L_2	G_3	Gl.	GlG	LG_2	G_4	Gl.	GlG_2	G_5	Gl.	GlG_2	LG_4	C
G	0 / tr.	+± / ++	± / +±	+ / ±+	± / ++	+± / +++±	±+ / ++	+± / ++	± / +±	±+ / ++	±+ / ++	+± / ++	+ / ++	+ / ++	+ / ++	±+ / ++	+ / ++	±+ / +++
L	± / +±	0 / 0	+ / ++	0 / +	± / +±	0 / ±+	±+ / ++	0 / ±	± / +±	tr. / +	+ / +±	0 / +	tr. / +±	+ / +±	0 / ±	± / +±	± / +±	+ / ++
Gl.	++ / +++	+± / +++±	++ / +++±	++ / +++	++ / +++	+ / ++	++ / +++	f. tr. / +	++ / +++±	++ / +++±	+± / +++±	f. tr. / +	+± / +++±	+± / +++±	tr. / +	+± / ++	++ / +++	++ / +++
LG	++ / +++	++ / +++±	+± / +++	0 / 0	++ / +++±	+± / +++±	++ / +++±	++ / +++	± / +±	+± / +++±	++ / +++±	++ / +++	+± / +++±	+± / +++±	++ / +++	+± / +++	+± / +++	++ / +++±
G_2 No. 1	± / +++±	+± / ++	f. tr. / ±	+ / +±	0 / ±	+± / ++	0 / ±	+± / ++	± / +±	tr. / +	tr. / ±	+± / ++	±+	± + / ++	± +	±+ / ++	± +	±+ / +++
G_2 No. 2	+ / ++	± / ++	f. tr. / ±	+ / +±	0 / tr.	+ / ++	0 / ±	+ / ++	± +	+ / ++	0 / tr.	+ / ++	± +	f. tr. / tr.	f. tr. / tr.	± +	f. tr. / tr.	±+ / +++±
LG_3 No. 1	± + / +++	+± / +++±	± / +++±	+± / +++	+± / ++	+ / +++	+ / ++	+± / +++	± +	0 / +	±± / +++	+ / +++	± / +±	+± / +++±	+ / +++	f. tr. / +±	+± / ++	+± / +++±

that of the precipitin tests, cannot be explained on the basis of our present information and this question will require further study. It may be worth noting that with the precipitin tests, too, reactions of higher specificity may be obtained after absorption with heterologous antigens (9).

The investigations are being continued along the lines indicated in our previous communication(1).

SUMMARY

In continuation of previous studies immune sera for azoproteins made from aminobenzoyl dipeptides and tripeptides were tested with various peptide azoproteins by precipitin tests and with nitrobenzoyl derivatives of peptides by means of inhibition reactions. When examined with the latter method, the immune sera exhibited a high degree of specificity and permitted the recognition of distinctions among peptides of similar structure.

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EPIDEMIC TREMOR, AN ENCEPHALOMYELITIS AFFECTING YOUNG CHICKENS*

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PLATES 51 AND 52

During the last 4 years there has appeared in New England flocks a disease of young chickens hitherto unrecognized. This disease was described in a preliminary report (Jones, 1932) as "An encephalomyelitis in the chicken." Its increasing frequency among commercial flocks in the last 2 years has made necessary the use of a more descriptive name. In view of the striking symptom which differentiates it from other nervous disorders, and because of its appearance in large numbers of chickens within a flock, the name "epidemic tremor of chickens" has been chosen. The present paper is a report of studies carried on in field epidemics and in the laboratory since its first appearance.

The disease has been transferred experimentally to normal chickens by intracerebral inoculation of suspensions of brain and spinal cord. During the course of twenty passages, the virulence has been materially increased. Whereas in early experiments only a few of the inoculated birds contracted the disease, in recent transfers it has not been unusual for an entire series to become infected. The average incubation period has likewise been shortened in the course of these passages, and the severity of the brain lesions has increased. The condition has many of the characteristics of a virus disease and has been tentatively classed as such.

Clinical Course

The first symptom observed in affected flocks is usually the constant trembling of certain individuals. Attention is called to these birds by the rapid vibration of the head, but on handling them, it is apparent that muscles in addition to those of the neck are affected to a variable degree. The tremor becomes aggravated when the birds are handled or when the flock is in any way excited, but tends to subside when the birds are undisturbed, and disappears in sleep. On further examination of a flock, other chickens less severely affected are often found. Such birds may exhibit a fine tremor which is scarcely noticeable until the bird is picked up. Ataxia is also a symptom which usually is seen in conjunction with tremor, but has occasionally been the only symptom to appear. When ataxia is the first manifestation of the disease, tremor may develop later. More frequently, ataxia appears simultaneously with, or at varying intervals subsequent to, the development of tremor. Those chickens which are affected by tremor alone are apparently not seriously incapacitated, since they walk about, and are able to eat and drink in spite of the constant head tremor. Even in the most advanced cases, in which a coarse tremor is constantly present, the birds are still able to peck at food, and do not appear greatly inconvenienced by their condition. All of the birds affected with tremor alone seem capable of survival for an indefinite period under laboratory conditions. Those chickens which have ataxia associated with the tremor, or ataxia alone are seriously handicapped in their efforts to move about, and as the ataxia is progressive, the birds ultimately are unable to reach sufficient food to maintain themselves even in confinement.

Pathology

The pathology of the disease was studied in detail in the birds received during the outbreak in 1931. We are indebted to Dr. M. M. Canavan of the Department of Pathology for painstaking examination of the brains and spinal cords of the birds of this series, and for the original description of the lesions of the nervous system. Similar detailed studies were made in birds from epidemics in 1932 and 1933, and in a series of inoculated birds. Routine examination of birds from field epidemics and of inoculated birds to correlate pathology with symptoms is now confined to sections of the brain alone.

Distribution of Lesions.—The characteristic lesions of the disease are microscopic and are found scattered throughout the brain and spinal cord. No lesions which could be detected in the gross have been found in the central nervous system or viscera. There have been no tumors noted, and no changes in eye coloring or vision.

In addition to the involvement of the nervous system, microscopic

lesions are found in the viscera of birds suffering from natural infections, but rarely in chickens which have been inoculated.

The distinctive lesions of the brain and spinal cord of birds from field epidemics consist of microscopic collections of neuroglia cells (Figs. 1 and 2). These are composed of macroglia and oligodendroglia with an occasional microglia cell. They are found clustered around capillaries. Cells are seen in mitosis in these foci, which indicates the proliferative nature of the lesion. Foci of glia cells are often found near the ventricles of the brain in association with degenerating nerve cells. Similar lesions are found thoughout the brain and spinal cord. In the earlier series examined, they appeared to be more numerous in the cerebrum than in the cerebellum, but in more recent cases the lesions of the cerebellum seem to be of greater severity. The number of foci is also greater, and the foci are larger than in the earlier series. This is particularly true in the inoculated birds. Perivascular infiltration around the larger vessels of the brain is also found in many instances, but varies greatly in severity. Degeneration of Purkinje's cells is often severe.

The lesions of the brain and spinal cord of inoculated birds are similar to those described above. In many cases, however, they are far more severe than anything encountered in spontaneous cases of the disease. The lesions are largely perivascular. The focal collections of neuroglia cells, which are the typical lesions, are most often found surrounding the small vessels in the deeper portions of the brain. The cells composing these collections are macroglia, oligodendroglia, and microglia, as in the lesions of spontaneous cases, and in addition there is often an astrocyte involved (Figs. 3, 4, and 5).

In inoculated birds infiltration around the larger vessels of the brain is sometimes extremely severe. In certain cases this is largely lymphocytic in character. Lymphocytic infiltration is also present at times around the meningeal vessels. It is apparently greater around veins than their accompanying arteries.

Studies of the peripheral nerves have revealed neither gross nor microscopic lesions.

The typical lesions of the visceral organs of spontaneous cases are microscopic foci of infiltration with cells of the lymphoid series. The foci are of two types, one rounded and sometimes sharply circumscribed and encapsulated, the other, irregular, with ill defined boundaries. The circumscribed lesions are most commonly and strikingly found between the alveoli of the pancreas, often in the vicinity of blood vessels (Fig. 6). The blue staining of their cells is pale in contrast to the irregular pinkish islands of Langerhans, and the intense blue of the cells of the alveoli. Similar areas are also found in the spleen and infrequently in the heart. The infiltrations in the spleen vary greatly in appearance, and comparison of sections from the spleens of normal and diseased birds has led to the conclusion that in many cases it is impossible to distinguish between normal and pathological accumulations of cells in this organ.

The rounded, circumscribed lesions are largely composed of cells resembling the lymphoblasts. The nuclei are large, with coarse granules of chromatin. There are mitotic figures in many of the lesions, and numerous pycnotic nuclei. The areas are often surrounded by a narrow band of connective tissue, and certain of these areas are joined by irregularly shaped infiltrations of cells of similar type. The irregular areas of infiltration are found most frequently in the heart. They also occur in the pancreas and spleen and are occasionally present in the liver, kidney, lung, ovary, and other organs. They are composed chiefly of cells resembling large lymphocytes, and no mitotic figures have been seen among them. In the heart, such infiltrations often push apart the muscle bundles (Fig. 7). There seems to be no degeneration of the muscle fibers, but in some cases the heart shows other evidences of chronic inflammation.

Since lymphoid cell infiltrations of similar nature are encountered in many pathological conditions in chickens, these lesions are not considered in any way distinctive of epidemic tremor, although the infiltration in the pancreas is unusual. The fact that they do not occur in the inoculated birds may indicate a more strictly localized reaction to the infective agent, possibly due to its mode of introduction.

Epidemiology

Our first experience with the disorder was in May, 1930, when nine Rhode Island Red chickens about 2 weeks of age were brought to us from a commercial flock. These birds were affected with a fine tremor which did not abate during a period of observation extending over 3 months. The group was abnormally excitable. No ataxia developed among them, nor to our knowledge was there any ataxia in the flock from which they were taken.

The disease was not seen again until April, 1931, when eleven Rhode Island Red chickens were brought to the laboratory from a different source. Six of the birds were 4 weeks old and had been kept on the farm where they were hatched. The other five birds were from the same stock but hatched 1 week later. They had been sold as day old chicks and consequently had been under different environmental conditions since hatching. The older birds were less severely affected than the younger ones. All showed tremor but of varying degrees of severity. Some of the birds in both groups were ataxic on their arrival in the laboratory and some developed ataxia later.

In January, 1932, severe outbreaks occurred on one poultry farm in early hatches of eggs from one source. Forty-two birds from 2 to 6 weeks of age were sent to the laboratory. It was said that certain birds on this farm had exhibited tremor on the 2nd day out of the incubator. All of these birds exhibited tremor when sent to us, and many of them were likewise ataxic.

The occurrence of a similar condition in several other flocks was reported to us during the spring of 1932, but no specimens were sent to the laboratory until May when forty chickens were sent in from a flock unrelated to that in which the January outbreak had occurred. All but one of these chickens were ataxic, and twenty-seven of them were also affected with tremor.

The epidemics of 1930, 1931, and spring of 1932 all occurred in Massachusetts. After the May, 1932, epidemic, no cases were heard of until November, 1932, when a serious epidemic was reported in New Hampshire. This was followed by outbreaks in other New England states until by the first of June, 1933, there had been reported to this laboratory as having occurred during the winter and spring hatching season of 1932–33, eight epidemics from Massachusetts, four from New Hampshire, two from Maine, and one from Connecticut. There have doubtless been other affected flocks which have not come to our attention. No cases of the disease have been reported during the months from June to November, 1933. Affected birds in the majority of these flocks have exhibited tremor, ataxia, or both. Birds from two flocks were affected with ataxia only.

From epidemics in 1932, there was some evidence that the disease was more frequent among chicks of the first hatch of the season than in later hatches. Further experience, however, has proved that the appearance of the disease in relation to hatch is a matter of chance. Certain epidemics have been confined to the first hatch of the season; other outbreaks have affected the first three hatches; while still others have appeared in the middle or toward the end of the breeding season, and have affected from one to three hatches.

There have been some cases of recurrence of the disease in flocks in the 2nd year. No recurrence has been noted for 2 successive years in the flock involved in the 1931 epidemic.

The disease may appear in young chickens during the 1st week or subsequently up to 5 or 6 weeks of age. In one case tremor is said to have appeared at 3 days, and in another, at 5 days. The usual time of onset, however, is at 3 weeks of age. No epidemics have been reported among adult birds. Epidemics have occurred in Rhode Island Red, New Hampshire Red, Barred Plymouth Rock, and White Plymouth Rock flocks.

Etiology

Early in the study of these outbreaks, experiments were undertaken to determine the nature of the disease. Efforts were made to transmit the conditions to normal birds and to cultivate an organism from the tissues of diseased birds. In addition, the possibility of the disease being a nutritional disorder, or a form of food poisoning was considered.

Chemical analysis for calcium and phosphorus was made of the blood of two birds with severe tremor. The analysis showed 11 mg. calcium and 8.7 mg. phosphorus which was considered[1] a normal value for young chickens.

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[1] We are indebted to Dr. Joseph C. Aub for the calcium and phosphorus determinations and for their interpretation.

No evidence of rickets has been found.

Influence of Vitamins.—The occurrence of nervous disorders thought to be due to the absence or deficiency of various vitamins in the diet has been reported by several investigators (Hughes, Lienhardt, and Aubel, 1929, Hogan and Shrewsbury, 1930, Wolf and Pappenheimer, 1931). To determine whether vitamin deficiency was a cause of epidemic tremor, certain affected chickens sent to us during the 1931 epidemic were given a diet supplemented with whole milk, lettuce, hard boiled egg, and a commercial vitamin B concentrate. No influence of diet was discerned.

Attempts to Cultivate an Organism.—All attempts at cultivation of an organism of etiological significance in the disease have been unsuccessful. Cultures on agar slants were made of brain suspensions used for inoculation. With the exception of occasional obvious contaminants, the cultures were negative. Cultures of brain and spinal cord from birds received from field epidemics were made on a variety of media including broth, agar, agar plus normal chicken serum, blood agar, and chopped meat media. Cultures were also made of heart's blood, liver, spleen, and pancreas from a series of ten spontaneous cases. Broth, agar, agar plus serum, and chopped meat media were used. No organism of etiological importance was isolated in either of these series.

Contact Experiments.—Attempts to transmit the disease to normal birds through contact with affected birds were made in three epidemics. Normal and diseased chicks were kept in the same cages with every opportunity for contamination of the feed and drinking water and exposure to droppings. In one experiment a suspension of brain from a diseased bird was mixed with the drinking water, and some of this suspension was likewise introduced into the nares by means of a fine pipette. No disease developed in any of the exposed birds, nor were lesions found in the brain on microscopic examination.

Tests of Suspected Feed.—The grain supply naturally was suspected by poultry men to be the cause of the disease in several of the early outbreaks. Experiments were carried out (1) to test the grain for contamination with the infective agent, (2) to discover whether the composition of the mash was at fault, and (3) to see whether a toxic substance was produced in mash when stored in a warm place. A commercial broiler ration from farms where it was being fed to young chicks among which the disease had appeared was used in these experiments. Groups of normal chickens in the laboratory were fed this ration (a) without autoclaving; (b) after autoclaving at 15 pounds pressure for 20 minutes; (c) without autoclaving and after storage in a very warm room. None of the fifty chickens in these experiments developed the disease. Microscopic examination was made of the brain from nine birds. No lesions were found.

Tests were also made for the presence of spore-bearing anaerobes in the feed. None were found.

Inoculation Experiments.—Attempts were made in 1930, when the first affected chicks were studied, to transmit the disease to normal

chickens by intraperitoneal inoculations of suspensions of intestine, liver, spleen, and brain. A portion of the intestine with its contents was also made into a suspension in normal saline and given *per os* to a normal chicken. The inoculated birds were kept in the laboratory for 112 days. They developed no symptoms during that time. At autopsy the gross findings were negative in every case. The brains were later studied microsopically. Unequally stained nerve cells were found, slight perivascular infiltration, and rare, small, focal collections of glia cells. No lesions were found in the spinal cords of these birds. In view of the results of later inoculation experiments in which inoculated birds were found to have brain lesions without having shown symptoms of disease, it is possible that these birds acquired the disease, but in so light a form that they exhibited none of the typical signs.

Since these early experiments, few attempts have been made to transmit the disease by other than intracerebral inoculation of suspensions of brain and spinal cord. The following experiments with inoculation by other routes and with other organs may, however, be summarized:

Suspensions of liver, spleen, pancreas, and gall bladder from birds in the 1932 epidemics were inoculated subcutaneously into a small series of normal chicks. No symptoms were observed in any of the birds so inoculated, nor were any lesions found in the brain. Suspensions of brain and spinal cord of birds in the 1932 epidemic were also given to a group of normal chickens *per os*, intraperitoneally, intracerebrally, and by the two latter routes combined. No symptoms were observed in the inoculated birds and no brain lesions were found.

More recently, intraperitoneal and intracerebral inoculations have been made with a suspension of spleen from a bird inoculated with the eighteenth passage of the virus. As a control for the presence of virus in the bird, a suspension of brain from the same bird was inoculated intracerebrally into four birds. Brain suspension was likewise inoculated intraperitoneally into four birds. None of the seven birds receiving spleen suspension developed symptoms of disease after 5 weeks. Occasional small brain lesions were found in two of those inoculated intracerebrally, and some perivascular infiltration was found in the brain in two of these inoculated intraperitoneally. No lesions were found in the other three birds. Three of the four chicks receiving brain suspension intracerebrally developed symptoms in 27 to 33 days after inoculation. The fourth bird exhibited typical brain lesions. None of the birds inoculated with brain suspension intraperitoneally showed symptoms after 5 weeks. Small foci of infiltration were found in the brains of three of these birds. The fourth was negative.

Intracerebral inoculation of normal birds with suspensions of brain, spinal cord, or both, from diseased birds has proved to be an effective mode of transmission. By this method the disease has now been transferred through twenty successive passages. The following technique was used in the first eight passages and occasionally in some of the later transfers.

Diseased birds were killed by chloroforming, and the brain and spinal cord (if used) removed aseptically. The tissue was ground in a sterile mortar, and normal saline added to form approximately a 1:10 suspension by volume. The coarser particles were allowed to settle, and the supernatant fluid drawn into a tuberculin syringe with a 25 gauge needle. Inoculation was made into the cerebrum, 0.1 cc. to each bird. This procedure was later modified by grinding the tissue with sterile sand, and after centrifugalization, the supernatant fluid was used for inoculation. This method is now used for routine inoculation.

During the outbreak in January, 1932, the intracerebral inoculation of brain and spinal cord of affected chickens into normal chickens was first undertaken. Material from twenty-seven chickens in the January and May epidemics of that year was used in this experiment. Brain, or spinal cord, or both from each bird was made into a suspension, as previously described, and four or more young Rhode Island Red chickens were inoculated with each suspension. A total of 91 chickens were inoculated, of which three developed typical tremor and ataxia. Each of the three chicks to develop symptoms had been inoculated with material from a different bird. Microscopic examination was made of the brains of forty-five of the chickens which had been inoculated but had developed no symptoms of disease. Lesions were found in two, each in a different series. Of the five birds with either symptoms or lesions, two had been inoculated with a suspension of spinal cord, two with a suspension of brain, and one with a suspension of combined brain and spinal cord. The incubation periods in the chicks showing symptoms were 29, 30, and 35 days. The fact that only five birds contracted the disease out of 91 chickens inoculated suggests that the active infective agent was present in the brain in only a small proportion of the chickens at the time they were used in the inoculation experiments.

In subsequent passages, a higher proportion of successful inoculations resulted, and the average incubation period was shortened.

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The brain and cord of each of the three chickens which had developed tremor and ataxia after inoculation in the first passage were used for further inoculations. The results of this passage and of the subsequent passages are presented in Table I. Rhode Island Red chickens were used in the majority of these experiments, and White Leghorns in the remainder. Age of birds at inoculation varied from 2 days to 15 days.

These experiments may be summarized as follows:

Epidemic tremor of chickens has been transmitted through twenty passages by intracerebral inoculation of suspensions of brain, spinal cord, or the two combined.

In those series of inoculations in which at least one chicken showed disease, the number of infections has varied from 25 per cent in the early passages to 100 per cent in many of the later ones. The incubation period varied from 6 to 44 days, with the largest number of individuals showing symptoms during the 3rd and 4th weeks.

The infective agent has been shown to be present in saline suspensions of both brain and cord. After centrifugalization, it is present in the supernatant fluid as well as in the sediment of such suspensions. The infective agent in the supernatant fluid is viable for at least 6 hours at room temperature, for 18 hours at 37°C., and for 48 hours at 5°C. Its survival in 50 per cent glycerine will be discussed later.

Combined intracerebral and subcutaneous or intraperitoneal inoculations proved to be no more effective than intracerebral inoculations alone.

The addition of normal chicken serum, 2 per cent starch in salt solution, or minced chicken embryo to the suspensions failed to increase the proportion of infections.

The addition of testicular extract did not shorten the incubation period nor increase the proportion of infections over that of the control group in the one experiment in which it was tried.

Infectivity of Filtrates.—Epidemic tremor was early recognized as having many of the characteristics of a virus disease. A number of experiments have been undertaken to test the filter-passing ability of the infective agent in the brain. Since some of this work is still in progress, a detailed report will be made later. Results of completed experiments may be briefly summarized as follows:

Seitz filters were used in eleven experiments. 95 chickens were

TABLE I

Summary of Nineteen Intracerebral Passages of Epidemic Tremor in Chickens

Passage No.	No. of chicks inoculated	Date	Inoculation	Results Cases with typical symptoms	Cases without symptoms Examined microscopically	Cases without symptoms Lesions present	Incubation period (days)
		1932					
2	4	Feb. 24	1:10 suspension* brain 391	0	1	0	
	4	Feb. 24	1:10 suspension cord 391	0	1	0	
	10	Mar. 3	1:10 suspension brain 399	2	5	0	28, 29
	11	Mar. 3	1:10 suspension cord 399	0	2	1	
	5	June 18	1:10 suspension brain 641	0	3	1	
	6	June 18	1:10 suspension cord 641	0	3	0	
3	5	Apr. 6	1:5 suspension brain 516	0	1	0	
	5	Apr. 6	1:5 suspension cord 516	1	0		40
	3	Apr. 6	1:5 suspension brain 518	0	1	0	
	5	Apr. 6	1:5 suspension cord 518	1	0		34
4	4	May 13	1:5 suspension brain and cord 600 This suspension plus equal volume: (a) 2% suspension starch in normal saline	0	1	0	
	4		(b) Normal chicken serum	0	1	0	
	4		(c) 5 day chick embryo (minced)	0	1	0	
	4	May 27	1:10 suspension brain 592	1	1	0	32
	3		Inoculated intracerebrally	0	1	0	
	3		Inoculated intracerebrally and intraperitoneally	0	1	0	
	3	May 27	1:10 suspension cord 592	0	1	0	

No.	Date	Material inoculated					
5	June 18	1:10 suspension brain 661	4	2	2	0	24, 30
		1:10 suspension cord 661	5	0	2	0	
6	July 20	1:10 suspension brain 694	5	2	0		
		1:10 suspension cord 694	5	0	1	0	31, 41
	July 20	1:10 suspension brain 695	4	1	0		
		1:10 suspension cord 695	4	0	1	0	41
7	Aug. 20	1:10 suspension brain and cord 721	11	9	0	0	15, 16, 20, 21, 21, 34, 34, 36, 44 ...
	Aug. 20	1:10 suspension brain and cord 722	8	3	4	4	30, 30, 33
	Sept. 24	1:10 suspension brain and cord 756. Combined intracerebral and intraperitoneal inoculation	10	4	6	5	20, 23, 33, 44
	Oct. 5	Inoculated as above with brain and cord 756. Reinoculated intracerebrally with 1:10 suspension brain and cord 758	10	6	4	1	23, 27, 28, 29, 37, 41
	Sept. 24	1:10 suspension brain and cord 746. Combined intracerebral and subcutaneous inoculation	5	4	1	0	20, 29, 34, 35
8	Sept. 26	1:10 suspension brain and cord 757	3	1	2	0	17
		Supernatant fluid from this suspension after 5 min. centrifugalization	5	3	2	2	20, 31, 32
		Sediment after centrifugalization	3	2	0	0	
	Oct. 10	1:10 suspension brain and cord 747	5	0	0		23, 30
9	Oct. 21	1:10 suspension brain and cord 786 and 818	5	4	1	0	25, 27, 28, 38
		Supernatant of above suspension after 10 min. centrifugalization	5	1	4	2	26
	Oct. 25	Supernatant of 1:10 suspension brain and cord 797 and 793	2	2	2		23, 24
	Nov. 10	Supernatant of 1:10 suspension brain and cord 790	5	3	2	2	23, 25, 27
	Nov. 11	Supernatant 790 after 24 hrs. refrigeration	4	2	2	2	22, 26

* All suspensions were made in normal saline except as noted.

TABLE I—*Concluded*

Passage No.	No. of chicks inoculated	Inoculation	Results			Incubation period
			Cases with typical symptoms	Cases without symptoms		
				Examined microscopically	Lesions present	*days*
		1932				
	2	Nov. 18 1:10 suspension brain and cord 836 and 846 Intracerebral and subcutaneous inoculation	2			10, 19
10	4	Dec. 1 1:10 suspension brain 837 after 6 hrs. at room temperature	3	1	1	12, 13, 13
	2	Dec. 2 Supernatant of 1:10 suspension brain 838	1	1	1	12
	1	Dec. 8 Supernatant of 1:10 suspension brain and cord 903 and 907	1			26
	1	Dec. 9 Supernatant as above after 18 hrs. at 37°C.	1			25
	2	Dec. 12 1:10 suspension brain and cord 905	2			24, 26
11	3	Dec. 14 Supernatant of 1:10 suspension brain and cord 930	3			20, 20, 21
	3	Dec. 15 Supernatant of 1:10 suspension brain and cord 929	2			19, 35
	3	Dec. 16 Supernatant of 1:10 suspension brain and cord 926	3	1	1	19, 19, 35
	3	Dec. 19 Supernatant of 1:10 suspension brain and cord 925	2	1	0	18, 18
		1933				
12	4	Jan. 13 Supernatant of 1:10 suspension brain and cord 1033 and 1038	3	1	0	25, 25, 26
	3	Jan. 17 1:10 suspension brain 957	2	1	0	22, 27
13	4	Feb. 10 1:10 suspension brain 1113	4			20, 20, 20, 20
	3	Feb. 24 1:10 suspension brain 1121	3			19, 21, 27

14	2	Mar. 8	Supernatant fluid of 1:10 suspension brain 1220 and 1228	1	1	1	15
	5	Mar. 8	Supernatant fluid of 1:10 suspension brain 1218, 1224, and 1226	4	1	1	16, 20, 21, 27
	3	Mar. 28	Supernatant fluid of 1:10 suspension brain 1244 and 1245	2	1	1	20, 20
15	9	Apr. 29	Supernatant of 1:10 suspension 1272	9			21, 23, 24, 30, 33, 33, 40, 41, 42
16	8	June 21	Supernatant of 1:10 suspension brain 1398, 1424, and 1426	2	6	3	29, 33
17	3	July 24	1:10 suspension brain 1512	3	3	3	21, 21, 28
	5	July 29	Supernatant of 1:10 suspension brain 1475 and 1481 after 48 hrs. in refrigerator	2			16, 37
18	4	Aug. 31	Supernatant of 1:10 suspension brain 1522, 1524, and 1530	4	1	1	15, 19, 21, 25
	5	Sept. 8	Supernatant of 1:10 suspension brain 1523 and 1528 after 48 hrs. in refrigerator	4			26, 30, 30, 33
19	4	Oct. 7	Supernatant of 1:10 suspension brain 1583	3	1	1	27, 28, 33
20	3	Nov. 9	Equal parts of hormone broth and supernatant of 1:10 suspension in hormone broth of brain 1622 and 1624	3			6, 6, 36
	4		Equal parts testicular extract and supernatant of 1:10 suspension in hormone broth of brain 1622 and 1624	2			7, 22

inoculated with bacteriologically sterile filtrates. Three birds in three different experiments developed the disease, and eleven others in eight different experiments had typical microscopic brain lesions.

Berkefeld filters have also been used in a number of experiments. Typical disease as well as brain lesions have followed the inoculation of Berkefeld N filtrates in two experiments. Inoculation of Berkefeld W filtrates has thus far failed to produce disease.

Survival of the Infective Agent in Glycerine

Infective brain tissue preserved in 50 per cent glycerine for 6, 47, and 69 days has been used to inoculate three series of four chicks each. All of those inoculated with the 47 and 69 day material became ataxic. One of those inoculated with the 6 day material developed disease; two had typical brain lesions; the fourth was normal. The survival of the virus in glycerine for a period of over 2 months has thus been demonstrated.

Attempts to Demonstrate Transmission of the Infective Agent through the Egg

The occurrence of epidemic tremor in chicks less than a week old suggests that the disease may be transmitted through the egg.

A series of inoculations was made in 1933 with brain tissue of embryos from a stock in which epidemic tremor had appeared in the preceding hatch. Unfortunately the disease died out after its first appearance, and the completely negative experiment was hence inconclusive. It is planned to repeat this work.

Breeding experiments were likewise undertaken. A small number of chicks, offspring of affected birds, have been raised in the laboratory. None of these developed disease, nor were they immune at 6 weeks of age to intracerebral inoculation of infective brain.

A serious outbreak of epidemic tremor in chicks of known parentage at an experimental farm[2] in November, 1932, provided an opportunity to carry on breeding experiments on a large scale. The disease appeared only in the first hatch of the season and affected approximately 50 per cent of 400 chicks brooded. Pedigree hatching was then instituted, but no disease developed in chicks from these matings. Breeding pens from these birds and from the survivors of the affected

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[2] We are indebted to the Department of Poultry Husbandry of the New Hampshire Agricultural Experiment Station for the opportunity to observe and the permission to report these experiments.

birds of the first hatch were saved, and all eggs were incubated. No disease appeared in them up to January, 1934. A detailed report of these experiments will be published at a later date.

There is no available evidence that transmission takes place through the egg.

Controls

All of the Rhode Island Red chickens used in laboratory inoculations were obtained as day old chicks from one commercial hatchery. Many hundreds of chicks obtained from this source from time to time in lots of twenty-five or fifty have likewise been used for other experiments in progress in the laboratory throughout the course of the work with epidemic tremor. One chick showed a fine head tremor on arrival at the laboratory, but on examination of the brain no lesions were found. The cause of the tremor in this case remains unexplained. Aside from this one questionable bird, there have been no spontaneous cases of epidemic tremor in our chickens.

Many groups of chickens have been kept as controls for series inoculated with epidemic temor, and the brains of many uninoculated birds have been examined for lesions in the course of these experiments, but no effort has been made to keep a control group for each series of experiments.

DISCUSSION

The occurrence of tremors and ataxia in chicks has been reported in association with a number of diseases of various etiology.

Pappenheimer and Goettsch (1931) in describing the clinical behavior of chicks suffering from "nutritional encephalomalacia" state that many of the birds before they were completely prostrated, became incoordinate and ataxic, and that clonic spasms of the legs and sometimes coarse tremors were also observed. The disease appeared in young chicks on a particular diet. The lesions were found chiefly in the cerebellum, but occurred not infrequently in the cerebrum, midbrain, and medulla as well. The essential lesion is described as ischemic necrosis followed, if the animal survives, by reparative organization of the dead tissue. Dunlap (1932) has also reported an ataxia of chicks associated with nephritis. This disease is apparently similar in its symptom complex to that described by Pappenheimer and Goettsch; namely, muscular incoordination, twitching or tremor of the head and legs, and retraction of the head. No microscopic studies of the central nervous system were reported, but changes in kidneys and proventriculus were noted, both grossly and microscopically. The uric acid content of the blood was increased, indicating impairment of nitrogenous metabolism. It was believed that this disease was of nutritional origin since it was correlated in field cases with high protein intake and forced feeding. It was stated that changes in feeding prevented new cases.

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EXPLANATION OF PLATES

PLATE 51

FIG. 1. Ventricular area in brain of spontaneous case showing focal collection of glia cells. Cresylecht violet stain. × 65.
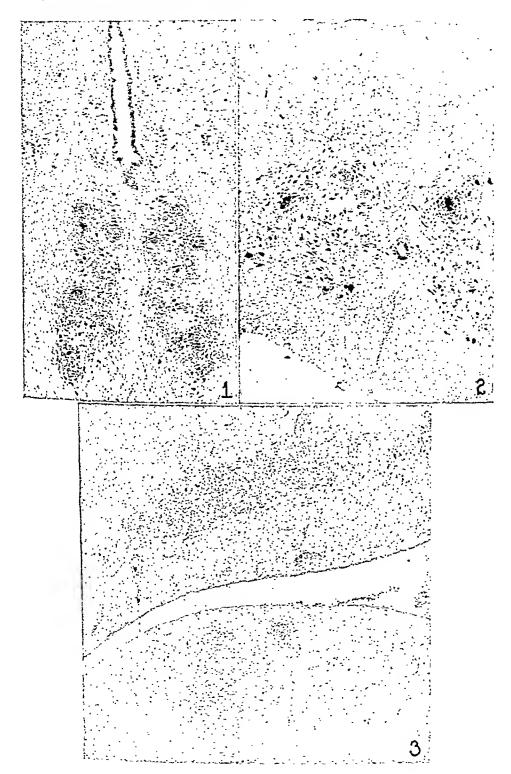
FIG. 2. Spinal cord of spontaneous case. Note collections of cells in gray matter. Cresylecht violet stain. × 60.

FIG. 3. Optic lobe of inoculated bird. Note perivascular infiltration. Eosin-methylene blue stain. × 65.

PLATE 52

FIG. 4. Cerebellum of inoculated bird. Severe focal infiltration. Eosin-methylene blue stain. × 65.
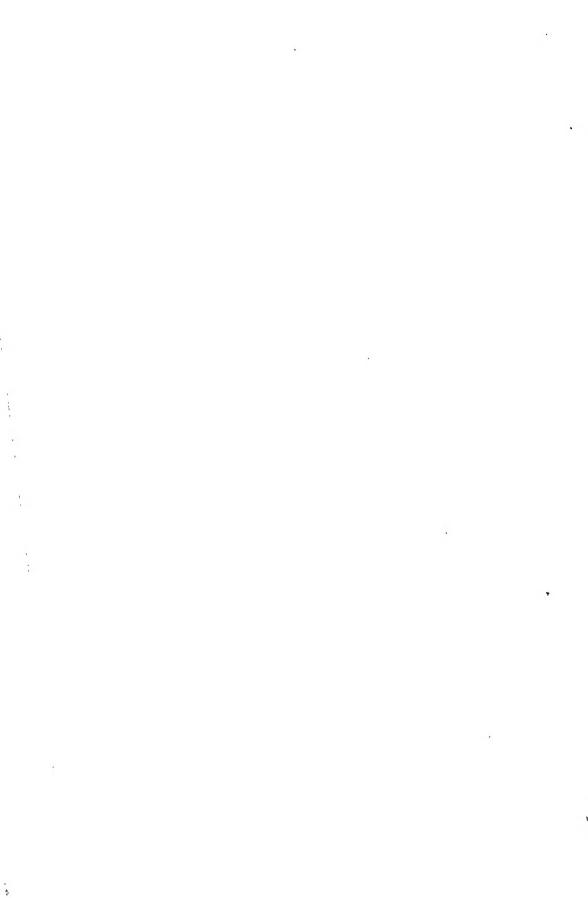
FIG. 5. Lesion in cerebellum of inoculated bird. Eosin-methylene blue stain. × 300.

FIG. 6. Pancreas of bird (spontaneous case) showing rounded collections of lymphoid cells. Eosin-methylene blue stain. × 300.
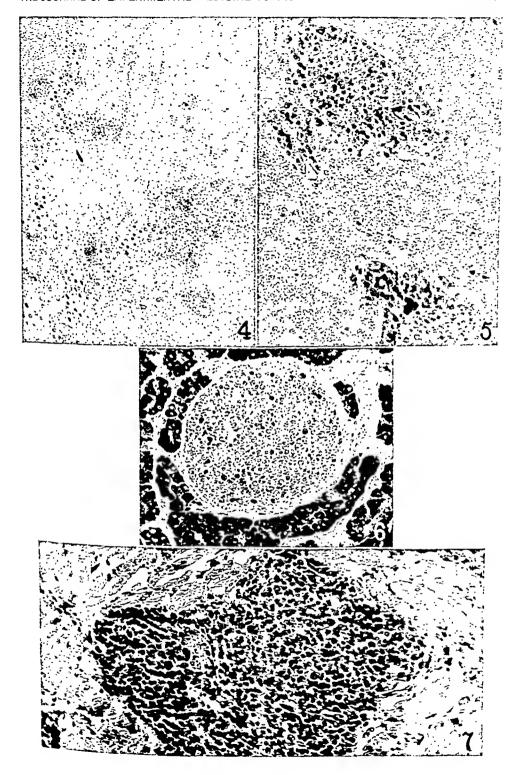
FIG. 7. Heart of bird (spontaneous case) showing area of infiltration of lymphoid cells. Hematoxylin and eosin stain. × 300.

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